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Antagonists that Demonstrate Species Differences in Neurokinin-1 Receptors

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SUMMARY

^{125}I-Bolton-Hunter-substance P (^{125}I-BH-SP) binding properties of three novel classes of neurokinin-1 (NK-1) receptor antagonists were investigated in tissues derived from humans, guinea pigs, and rats. ^{125}I-BH-SP was shown to bind to a single class of binding sites, with similar dissociation constants, K_d, in human astrocytoma cells (U-373 MG), human urinary bladder, guinea pig forebrain, guinea pig ileum longitudinal smooth muscle, rat forebrain, and rat duodenum. In each tissue preparation, known peptide agonists and peptide antagonists yielded potencies typical for a NK-1 receptor profile, with little difference in binding properties between the various tissues. However, when the three classes of compounds, heterosteroids, cyanines, and modified peptides, were tested for their ability to displace ^{125}I-BH-SP binding from the NK-1 receptor, very different binding profiles were observed. The heterosteroids were shown to be as much as 3 orders of magnitude more potent in tissues derived from rats than from humans or guinea pigs. A distinct species-dependent structure-activity relationship (SAR) was also observed for this class of compounds. Like the heterosteroids, the cyanines displaced ^{125}I-BH-SP with 10–30-fold higher affinity in rat tissues than in human and guinea pig tissues. However, the SAR generated by the cyanines was comparable in all tissues studied. The modified peptides, on the other hand, were up to 10–100-fold more potent in human and guinea pig than rat tissues, producing a SAR that differed between the various species. No differences in binding properties between central nervous system and peripheral tissues from the same species were seen with these compounds. These results provide evidence for species differences in NK-1 receptors in humans, guinea pigs, and rats. Because it is known that there exists great sequence identity between rat and human NK-1 receptors, it is hypothesized that key amino acid changes or different lipid environments within the transmembrane binding region of the receptor may account for the observed species difference. Furthermore, this study emphasizes that caution is necessary in the choice of species to be used in development programs targeted towards therapeutic entities in the NK-1 receptor antagonist area.

The NKs are a family of peptides that are characterized by a common carboxyl-terminal sequence, Phe-X-Gly-Leu-MetNH_2. At least three NKs, SP, NKA, and NKB, are believed to play important neuromodulatory roles in both the CNS and the peripheral nervous system. These actions are the result of the interaction of the NKs with membrane-bound receptors. There are known to be at least three receptors for the NKs, generally referred to as NK-1, NK-2, and NK-3 (1). The endogenous ligands for these receptors are believed to be SP, NKA, and NKB, respectively, based on their relative agonist potencies. Recently, three distinct NK receptors have been cloned and sequenced, and their properties appear to validate this classification (Ref. 2 and references cited therein).

To date, there has been no genetic evidence for further subclassification of these three receptors. However, recently developed classes of novel NK-2 antagonists have indicated notable differences in previously accepted NK-2-selective preparations. For example, the NK-2 antagonist Asp-Tyr-D-Trp-Val-D-Trp-D--Trp-ArgNH_2 has been reported to be 100 times more potent in murine fibroblast cells transfected with bovine stomach NK-2 receptor cDNA than in the hamster urinary bladder NK-2 binding assay (3). It has also been reported that the linear NK-2 antagonist AcLeu-Met-Gln-Trp-Phe-GlyNH_2, is active in the rat vas deferens ($pA_2 = 6.6$) and hamster urinary bladder ($pA_2 = 7.5$) NK-2 tissue bath bioassays but is inactive in the guinea pig trachea, rabbit pulmonary artery, and human urinary bladder tissues (4). A closely related analog, AcLeu-Asp-Gln-Trp-Phe-GlyNH_2, which differs by the replacement of methionine by aspartate, is active in all of the NK-2 tissue bath bioassays (4). These results appear to provide pharmacological evidence for heterogeneity of NK-2 receptors. It remains to be determined whether this heterogeneity is due to receptor subtypes or species-related differences.

There has been similar evidence for the heterogeneity of NK-1 receptors. The recently described nonpeptide quinuclidine NK-1 antagonist CP-96,345 shows a 35-fold potency difference

ABBREVIATIONS: NK, neurokinin; SP, substance P; ELE, eledoisin; ^{125}I-BH-SP, ^{125}I-Bolton-Hunter-substance P; CNS, central nervous system; BSA, bovine serum albumin; SAR, structure-activity relationship(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

in an [^{3}H]SP binding assay between bovine caudate and rat forebrain membranes (5). Nevertheless, no evidence was presented to indicate whether this was a subtype- or species-related receptor difference. The present study uses three novel classes of antagonists to explore NK-1 receptor binding differences in tissues of various anatomical origin from several species, in order to define a subtype- or species-dependent NK-1 receptor difference.

Materials and Methods

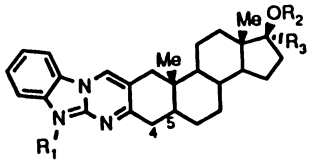
SP, ELE, NKA, NKB, and spantide were obtained from Cambridge Research Biochemicals; [D-Pro^2,D-Trp^{7,9}]SP, [D-Pro^2,D-Phe^7,D-Trp^9]SP, thiorphan, poly-D-lysine hydrobromide (M_r 30,000–70,000), bacitracin, leupeptin, chymostatin, and BSA were purchased from Sigma Chemical Company. The heterosteroids, cyanines, and modified peptides (Table 1) were synthesized by the Life Sciences Research Laboratories of Eastman Kodak Co. (Rochester, NY) or by the Department of Medicinal Chemistry of the Sterling Research Group (Rensselaer, NY). These compounds yielded spectral (^{1}H NMR and mass spectra) and analytical data (elemental analysis and/or high performance liquid chromatography) supporting their structural assignment and purity. The imidazole modification in **3a** was synthesized from D-tryptophan with retention of configuration at the α carbon. The thiazole modification in **3b** was synthesized from D-tryptophan with retention of configuration, but the compound was isolated as a single isomer of unknown stereochemistry at the carboxyl-terminal phenylalaninamide. The thiazole **3c** was isolated as a single isomer of unknown stereochemistry at the carbon α to the thiazole ring.

TABLE 1

Structures of novel NK antagonists

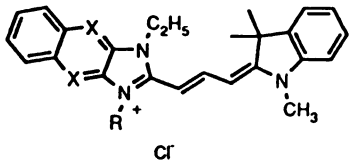
These include the heterosteroids (A), the cyanines (B), and the modified peptides (C).

A.



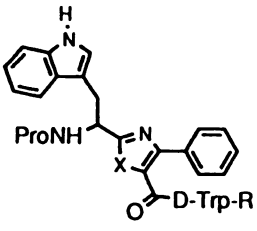
Compound	R_1	R_2	R_3	4-5
1a		H	$-C\equiv CH$	
1b		H	$-C\equiv CH$	Double bond
1c		H	$-CH_3$	
1d		CH_3	$-C\equiv CH$	
1e	CH_3^{+I-}	H	$-C\equiv CH$	

B.



Compound	X	R
2a	CH	$-C_2H_5$
2b	N	$-(CH_2)_3N(CH_3)_2$
2c[a]	N	$-(CH_2)_2OCOCH_3$

C.



Compound	X	R
3a	NH	$-$Leu-PheNH$_2$
3b	S	$-$Leu-MePheNH$_2$
3c	S	$-NHCH_2CH_2N(CH_3)_2$

[a] Tested as di-*p*-toluene sulfonate salt.

Preparations of membranes suspensions. Sprague-Dawley rats (200–300 g) (Taconic Farms, NY) and Dunkin-Hartley guinea pigs (250–500 g) (Hazleton, PA) were sacrificed, and the various organs were dissected rapidly and put on ice at 4°. Animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Sterling Research Group. Fresh tissue homogenates of rat and guinea pig forebrain (whole brain minus cerebellum), rat duodenum (minus mucosa), and guinea pig ileum longitudinal smooth muscle were prepared as previously described (6–8). Two post-mortem human urinary bladders (mainly detrusor muscle) were obtained, on dry ice, from the International Institute for the Advancement of Medicine (Exton, PA). Connective tissue and fat were dissected away from muscle tissue and membranes were prepared as described previously for hamster urinary bladder (8). The human urinary bladder membranes could be stored for at least 1 month at −70° without loss of binding activity. Tissue homogenates were resuspended in 50 mM Tris·HCl, pH 7.4, at 100–600 μg of protein/ml, for use in the binding assays.

^{125}I-BH-SP binding assay. Specific binding of ^{125}I-BH-SP to various membrane samples was examined in a final volume of 0.25 ml, in 96-deep-well polystyrene titer plates (Beckman). The binding assays were performed using a Beckman BIOMEK 1000 Workstation. Incubation medium consisted of 50 mM Tris·HCl, pH 7.4, containing 0.2 mg/ml BSA, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, and 1.2 mM $MnCl_2$. Routinely, the binding assay mixture contained 0.1 nM ^{125}I-BH-SP (2000 Ci/mmol) (Amersham) and duplicate samples of test compound at seven concentrations. Nonspecific binding was determined in the presence of 1 μM SP. Reactions were initiated by addition of membrane suspension (20–90 μg of protein) and were allowed to come to equilibrium for 30–90 min at 25°. The reactions were terminated by vacuum filtration over Whatman GF/C glass filters that had been presoaked in 0.1% polyethylenimine. Filters were immediately washed eight times with 1 ml each of 50 mM Tris·HCl, pH 7.4, 5 mM KCl, 120 mM NaCl. The radioactivity trapped on the filters was counted in a Packard Cobra γ counter.

The commercially obtained human astrocytoma cell line U-373 MG (HTB 17; American Type Culture Collection) was cultured as monolayers in polystyrene flasks, as described previously (9). Cells were maintained in modified Eagle's minimum essential medium containing a double concentration of all amino acids except L-glutamine (2.8 mM), a quadruple concentration of minimum essential medium vitamin solution, 10% (v/v) heat-inactivated horse serum, 200 IU/ml penicillin, and 200 μg/ml streptomycin. All chemicals used in the culture medium were obtained from GIBCO. The U-373 MG cells were prepared for experimentation by seeding of the cells on poly-D-lysine-pretreated 96-well plates (Corning), at 14,000–20,000 cells/well. Cells attached to the 96-well plates were maintained in culture medium and used for binding assays 2–3 days later. The binding affinities of various compounds were tested by competition assays against ^{125}I-BH-SP. The binding assay was carried out using a BIOMEK 1000 Workstation. In these experiments, the culture medium was replaced with balanced salt solution containing (in mM) NaCl, 115; KCl, 5.8; KH_2PO_4, 2.2; $MgSO_4$, 0.6; $CaCl_2$, 1.8; glucose, 5.0; and HEPES/NaOH, 25; pH 7.4; supplemented with 1.0% (w/v) BSA, 40 μg/ml bacitracin, and 1 μM thiorphan. The cells attached to the poly-D-lysine-pretreated 96-well plates were washed three times with 0.25 ml of buffer, and the assay mixture (100 μl), consisting of 0.1 nM ^{125}I-BH-SP and various concentrations of the compound, in triplicate, was added. When dimethylsulfoxide was used as a solvent, the final concentration never exceeded 0.5%. Control

experiments indicate that this concentration of dimethylsulfoxide has no effect on ^{125}I-BH-SP binding. Nonspecific binding was defined by the addition of 100 nM SP. The cells were incubated at 25° for 90 min, and unbound ligand was removed by washing the cells four times with 0.25 ml each of ice-cold balanced salt solution. The cells were detached from the plates by using 140 μl of 0.2% Triton X-100 containing 1 mg/ml BSA. Bound ^{125}I-BH-SP was determined by removing 100 μl of the Triton X-100/BSA solution. Typically, when the U-373 MG cells were grown to confluence, incubation with 0.1 nM ^{125}I-BH-SP yielded total binding of approximately 2500 dpm and nonspecific binding of approximately 400 dpm.

Equilibrium binding constants for specific ^{125}I-BH-SP binding to intact cells and various membrane preparations were determined using triplicate incubations and eight concentrations (0.01–1.0 nM) of the ligand. Apparent K_d and B_{max} values were obtained according to the method of Scatchard (10). The competition curve data were analyzed by computer-determined, nonlinear, least-squares, best fit of the data to the Hill equation (11), which determines the IC_{50} values and slopes (Hill coefficients). K_i values were determined according the method of Cheng and Prusoff (12). Membrane protein was determined by a modified Lowry procedure (13), using BSA as a standard.

Results and Discussion

To investigate the binding characteristics of NK-1 receptor antagonists in different tissues from several species, fresh membranes were prepared from CNS and peripheral tissues from rat and guinea pig. The tissues used in these studies have previously been reported to possess NK-1 receptor subtypes and include rat forebrain, rat duodenum, guinea pig forebrain, and guinea pig ileum (6–8). The human urinary bladder and the human astrocytoma cell line U-373 MG (9) were chosen as sources of human NK-1 receptors.

Equilibrium binding studies were carried out on the various tissue preparations, using ^{125}I-BH-SP to label the NK-1 receptor (Table 2). ^{125}I-BH-SP was shown to bind to a single class of binding sites in each preparation, with Hill coefficients not different from 1.0. Equilibrium binding constants, K_d, were found to be similar in each of the tissues used, and the absolute K_d values agree with previously published results for ^{125}I-BH-SP binding to the NK-1 receptor (8, 9, 14, 15). The number of binding sites, B_{max}, varied considerably between the various tissue preparations, with the human astrocytoma cells possessing the highest concentration of NK-1 receptors per milligram of protein.

The binding profile for various known peptide agonists and antagonists was investigated in these tissue preparations. The peptide agonist SP produced a concentration-dependent decrease in ^{125}I-BH-SP binding in each of the tissue preparations examined (Fig. 1A), yielding calculated K_i values of approximately 0.1 nM and Hill coefficients not different from 1.0 (Table 3). Each tissue possessed a typical NK-1 receptor profile, with an order of potency of SP ≫ ELE ≥ NKA ≥ NKB (Table 3), a binding pattern clearly distinct from those reported previously for NK-2 and NK-3 receptor subtypes (7, 8, 16). Inhibition of ^{125}I-BH-SP binding was also observed with the NK-1 receptor peptide antagonist spantide, which is shown graphically in Fig. 1B; calculated K_i values and Hill coefficients for each tissue preparation are presented in Table 3. The overall binding profile for known peptide antagonists was spantide > [D-Pro^2,Trp^{7,9}]SP > [D-Pro^2,D-Phe^7,D-Trp^9]SP in each of the tissue preparations.

Although subtle differences exist between the absolute K_i values obtained for the tissue preparations from rats, guinea pigs, and humans, there was no clear indication, from the binding profiles of peptide agonists and antagonists, of a difference in NK-1 receptor properties. However, when the three classes of compounds, heterosteroids, cyanines, and modified peptides (Table 1), were tested for their ability to displace ^{125}I-BH-SP binding, very different binding profiles were observed for the various tissue preparations (Table 4).

In the rat forebrain binding assay, the heterosteriods **1a** and **1b** inhibited ^{125}I-BH-SP binding with K_i values of approximately 25 nM. Scatchard analysis of compounds **1a** and **1b** showed a concentration-dependent increase in K_d and no change in B_{max} (17). Furthermore, these heterosteroids had no

TABLE 2

Equilibrium binding constants for ^{125}I-BH-SP in various tissue preparations

Binding constants were derived using ^{125}I-BH-SP concentrations from 0.01 to 1.0 nM and were analyzed by the method of Scatchard (10). Values are means ± standard errors from three separate experiments.

Tissue	K_d	B_{max}	Hill coefficient
	nM	fmol/mg of protein	
Human astrocytoma	0.21 ± 0.04	200 ± 28	0.996 ± 0.003
Human urinary bladder	0.06 ± 0.02	18 ± 1	1.044 ± 0.046
Guinea pig forebrain	0.18 ± 0.02	12 ± 1	0.970 ± 0.027
Guinea pig ileum	0.40 ± 0.05	110 ± 20	0.995 ± 0.001
Rat forebrain	0.08 ± 0.01	28 ± 3	0.970 ± 0.030
Rat duodenum	0.12 ± 0.03	64 ± 25	1.000 ± 0.020

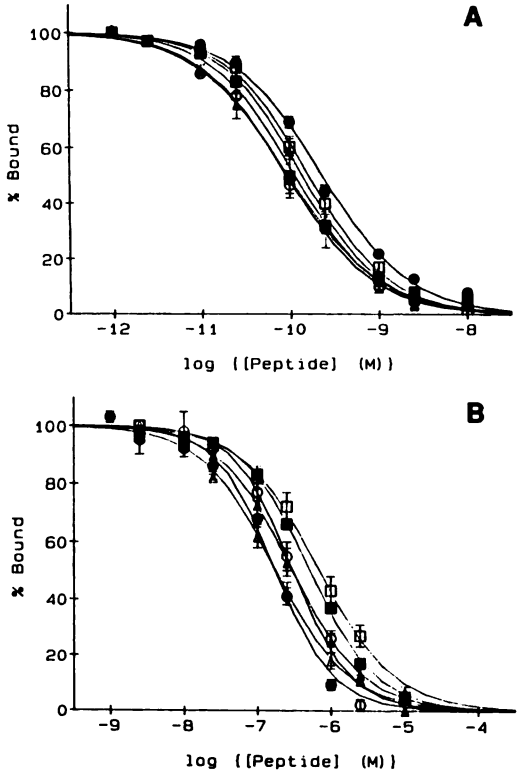


Fig. 1. Competition of ^{125}I-BH-SP binding by SP (A) and spantide (B) in various tissue preparations. The effect of SP and spantide on the binding of 0.1 nM ^{125}I-BH-SP was investigated in the following tissue preparations: ●, human astrocytoma; ○, human urinary bladder; ▲, guinea pig forebrain; △, guinea pig ileum; ■, rat forebrain; □, rat duodenum. Data shown are the means ± standard errors from three to five experiments. *Solid lines*, nonlinear best fits of the data to the Hill equation.

TABLE 3

Effects of NK peptide agonists and antagonists on ^{125}I-BH-SP binding

^{125}I-BH-SP binding to various tissue preparations was performed as described in Materials and Methods. IC_{50} values were determined by nonlinear, least-squares, computer-derived best fits of the data to the Hill equation (11), with each compound being assayed at seven concentrations. K_i values were derived using the Cheng-Prusoff (12) relationship. Results are from three to five separate experiments (means ± standard errors). Hill coefficients are given in parentheses.

	K_i values					
	Human		Guinea pig		Rat	
	Astrocytoma	Urinary bladder	Forebrain	Ileum	Forebrain	Duodenum
	nM					
Agonists						
SP	0.16 ± 0.01 (0.89)	0.04 ± 0.01 (0.92)	0.07 ± 0.03 (0.90)	0.11 ± 0.02 (1.02)	0.05 ± 0.01 (0.97)	0.10 ± 0.01 (0.97)
ELE	11 ± 1 (0.96	ND[a]	41 ± 11 (0.91)	46 ± 3 (0.84)	49 ± 9 (0.80)	23 ± 9 (0.84)
NKA	28 ± 5 (0.98)	6 ± 2 (0.82)	29 ± 3 (0.92)	88 ± 16 (0.83)	28 ± 4 (0.85)	20 ± 5 (0.80)
NKB	37 ± 3 (1.03)	8 ± 2 (0.80)	41 ± 10 (0.84)	62 ± 12 (0.93)	160 ± 29 (0.92)	130 ± 37 (0.83)
Antagonists						
Spantide	120 ± 10 (1.13)	120 ± 20 (1.13)	210 ± 40 (0.92)	150 ± 20 (0.86)	220 ± 4 (0.95)	430 ± 120 (0.89)
[D-Pro^2,D-Trp^{7,9}]SP	450 ± 50 (1.20)	270 ± 30 (1.09)	770 ± 60 (0.82)	500 ± 160 (0.93)	530 ± 30 (0.93)	490 ± 80 (1.04)
[D-Pro^2,D-Phe^7,D-Trp^9]SP	3100 ± 340 (1.16)	1600 ± 100 (1.17)	3700 ± 770 (0.96)	2900 ± 1200 (1.00)	1400 ± 40 (1.00)	1300 ± 400 (1.08)

[a] ND, not determined.

TABLE 4

Effects of heterosteroids, cyanines, and modified peptides on ^{125}I-BH-SP binding

^{125}I-BH-SP binding to various tissue preparations was performed as described in Materials and Methods. Competition curve data were analyzed by nonlinear, least-squares, computer-derived, best fits of the data to the Hill equation (11), which determines IC_{50} values and slopes (Hill coefficients, given in parentheses). Each compound was assayed at seven or eight concentrations. K_i values were derived using the Cheng-Prusoff (12) relationship. Results are from three to five separate experiments (means ± standard errors).

	K_i values					
	Human		Guinea pig		Rat	
	Astrocytoma	Urinary bladder	Forebrain	Ileum	Forebrain	Duodenum
	nM					
Heterosteroids						
1a	>25,000	>10,000	>10,000	>10,000	22 ± 5 (0.86)	27 ± 4 (0.83)
1b	>25,000	>10,000	>10,000	>10,000	21 ± 4 (0.81)	27 ± 5 (0.95)
1c	>25,000	>10,000	>10,000	>10,000	87 ± 8 (0.84)	160 ± 40 (0.80)
1d	>25,000	>10,000	>10,000	>10,000	>10,000	>10,000
1e	>25,000	>25,000	>25,000	>25,000	67 ± 9 (0.91)	82 ± 6 (0.95)
Cyanines						
2a	12,000 ± 4,000 (1.19)	1,900 ± 600 (1.19)	2,600 ± 400 (1.19)	1,900 ± 700 (1.05)	160 ± 40 (0.99)	150 ± 60 (0.93)
2b	>25,000	>25,000	>25,000	>25,000	580 ± 60 (0.95)	580 ± 110 (0.85)
2c	2,000 ± 300 (1.08)	4,100 ± 400 (1.06)	3,700 ± 1,100 (1.08)	6,100 ± 1,200 (0.90)	120 ± 10 (0.97)	130 ± 30 (0.81)
Modified peptides						
3a	53 ± 9 (0.98)	250 ± 80 (1.15)	460 ± 120 (0.94)	490 ± 150 (0.80)	2,900 ± 100 (1.24)	5,800 ± 600 (1.02)
3b	100 ± 40 (1.11)	180 ± 50 (1.09)	190 ± 30 (0.80)	350 ± 80 (0.80)	1,000 ± 100 (1.15)	1,800 ± 500 (0.84)
3c	>10,000	>10,000	>10,000	>10,000	1,600 ± 470 (0.99)	1,500 ± 500 (0.80)

effect on the rate of ^{125}I-BH-SP dissociation, compared with dissociation rates determined in the absence of compound (17). The Scatchard and dissociation results are consistent with a reversible competitive interaction of these compounds with the NK-1 receptor in rat forebrain. These same ^{125}I-BH-SP binding characteristics of the heterosteroids were observed in rat duodenum, suggesting that the NK-1 receptors found in rat CNS and peripheral tissues are similar. *In vivo* studies indicate that these heterosteroids produce a concentration-dependent inhibition of SP-induced plasma extravasation in rats, with IC_{50} values of approximately 1 mg/kg (17). In contrast to the rat tissues, the heterosteroids, including **1a** (Fig. 2A), were found

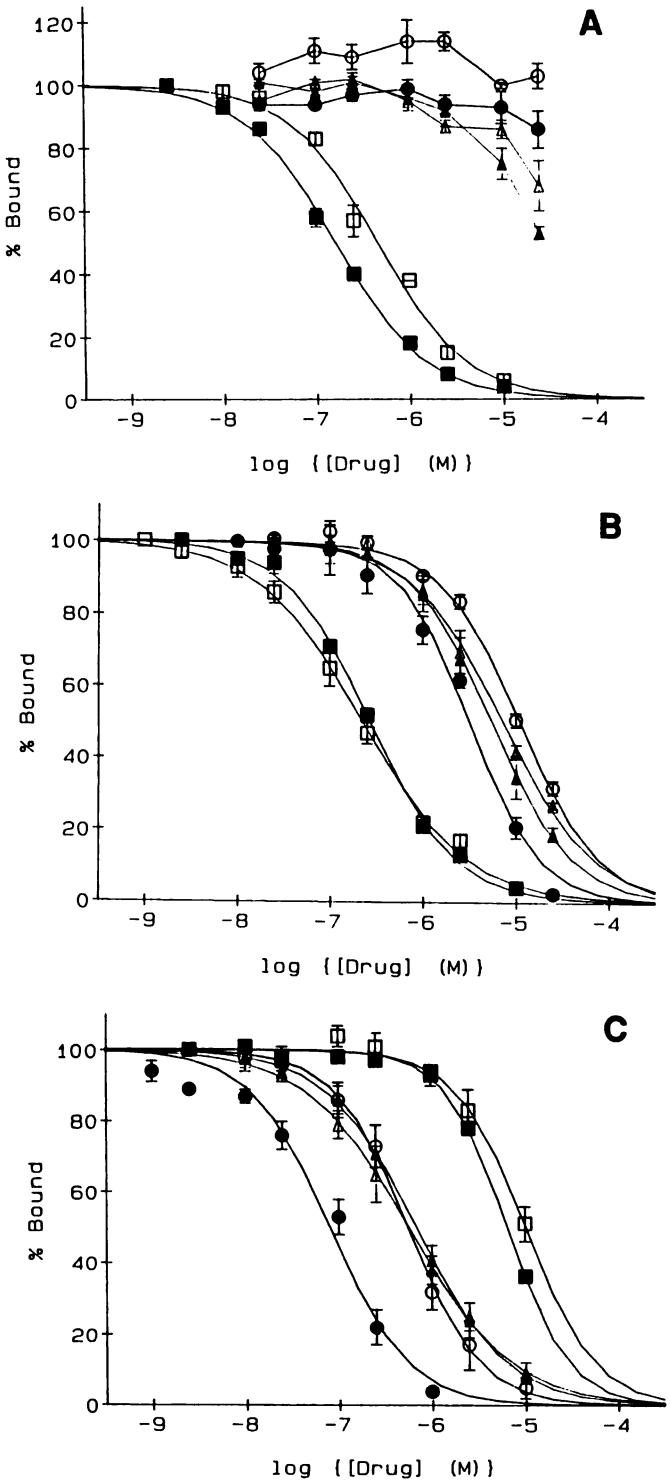


Fig. 2. Competition of ^{125}I-BH-SP binding by the heterosteroid **1a** (A), the cyanine **2c** (B), and the modified hexapeptide **3a** (C) in various tissue preparations. The binding of 0.1 nM ^{125}I-BH-SP was investigated in the presence of increasing concentrations of drug, in the following tissue preparations: ●, human astrocytoma; ○, human urinary bladder; ▲, guinea pig forebrain; △, guinea pig ileum; ■, rat forebrain; □, rat duodenum. Each data point represents the mean ± standard error from three to five experiments. *Solid lines*, nonlinear best fits of the data to the Hill equation.

to be inactive in inhibiting ^{125}I-BH-SP binding in guinea pig forebrain, guinea pig ileum, human astrocytoma, and human urinary bladder, at the highest concentration tested. Thus, absolute potency differences, of up to 3 orders of magnitude, exist in rat versus human and guinea pig tissues.

The SAR, based on the NK-1 receptor binding profile in the rat, was very sensitive to substitutions at the 17-position of the heterosteroid (Table 4). The heterosteroids shown in Table 4 were inactive in human astrocytoma, human urinary bladder, guinea pig forebrain, and guinea pig ileum. However, other heterosteroids[1] were found to possess low micromolar activity in human astrocytoma, guinea pig forebrain, and guinea pig ileum but were less active than compounds **1a** and **1b** in rat tissues. In general, this class of compounds possessed dramatically different SAR in human astrocytoma, guinea pig forebrain, and guinea pig ileum versus rat forebrain and rat duodenum. The varied NK-1 receptor structural requirements for heterosteroid activity suggest a species-dependent SAR, which appears to be independent of CNS versus peripheral tissue differences.

Like the heterosteroids, the cyanines, which include the imidazonaphthalene **2a** and the imidazoquinoxalines (18) **2b** and **2c** (Fig. 2B), displayed differences in binding to human astrocytoma, human urinary bladder, guinea pig forebrain, and guinea pig ileum versus rat forebrain and rat duodenum. However, these differences were not as dramatic as those found with the heterosteroids. Typically, the cyanines were 10–30-fold less potent in displacing ^{125}I-BH-SP binding in human and guinea pig tissues than rat tissues. Although the absolute K_i values differed in these tissues, the overall SAR generated in rat tissues was comparable to that found in guinea pig and human tissues. The order of potency was **2a** ≥ **2c** > **2b** in each of the tissue preparations. Furthermore, little difference in absolute K_i values was observed with the cyanines between CNS and peripheral tissue preparations from the same species.

The modified peptides displaced ^{125}I-BH-SP binding in the rat with an order of potency of **3b** ≥ **3c** > **3a** and an overall difference in K_i values of approximately 3-fold. In contrast to the two other classes of compounds, the K_i values for compounds **3a** (Fig. 2C) and **3b** were 10–100-fold lower in human and guinea pig tissues than in rat tissues, with an order of potency of **3a** ≥ **3b** > **3c**. Compound **3c** was inactive up to 10 μM in guinea pig forebrain, guinea pig ileum, human astrocytoma, and human urinary bladder, whereas micromolar activity was seen in the rat forebrain and rat duodenum. The overall range of K_i values for these compounds was >50-fold in human and guinea pig tissues. These differences in K_i values and order of potencies suggest that the modified peptides generate distinct SAR depending on the species used in the binding assays.

The three classes of compounds, heterosteroids, cyanines, and modified peptides, were used to demonstrate species differences in NK-1 receptors from humans, guinea pigs, and rats, whereas the peptide agonists and antagonists failed to discriminate between NK-1 receptors from these species. The heterosteroids and cyanines displaced ^{125}I-BH-SP binding with higher affinity in rat tissue preparations than in human and guinea pig tissues. These differences were most dramatic in the heterosteroid class of compounds, with absolute potencies differing by as much as 3 orders of magnitude. The modified

[1] K. C. Appell and B. J. Fragale, unpublished observations.

peptides, on the other hand, were up to 10–100-fold more potent in human and guinea pig than rat tissues. The SAR generated with the heterosteroids and modified peptides in the human and guinea pig tissues differed from that observed with the rat. In contrast, the cyanines were generally more potent in the rat by 10–30-fold but yielded a SAR that was comparable in all tissues studied.

In this study, distinct NK-1 receptor binding profiles in CNS tissues derived from rats, guinea pigs, and humans were observed and did not differ from those in peripheral tissues. An operational definition for these observations would be a species difference and not simply a receptor subtype. Receptor subtypes have generally been defined by ligands that display different binding properties in different tissue preparations from the same species (e.g., rat forebrain and rat duodenum). Under the assay conditions used here, no differences were seen between CNS and peripheral tissue preparations. Furthermore, Hill coefficients near 1.0 were observed in saturation and competition experiments, suggesting that ^{125}I-BH-SP is binding to a single class of binding sites in each of the tissue preparations examined or that SP and the compounds used in this study do not discriminate between receptor subtypes. Evidence against receptor subtypes was provided by a recent study suggesting that one mRNA species exists for the NK-1 receptor in both the CNS and peripheral tissues, at least in rats (19). Nevertheless, subtypes would not provide an explaination for the apparent differences observed between tissues derived from rats, guinea pigs, and humans. These differences were found between similar tissues from different species, thus providing evidence for a species difference in NK-1 receptors. Thus, based on the NK-1 receptor binding profiles, at least two clearly distinct groups of receptors exist, with the mouse[2] and rat differing from the human and guinea pig NK-1 receptors.

It is interesting to speculate about the origin of this observed species difference. Because the difference between assays is the source of the receptor preparation, it appears that the species difference must reside in the structure of the receptor protein or in the composition of the lipids in which the receptor protein is embedded. The structure of the receptor protein may differ in its primary structure, the amino acid composition. Recent evidence has suggested that there is >92% sequence identity between the human and the rat NK-1 receptors (20). In spite of this high level of identity, there are key amino acid differences between the human and rat NK-1 receptors, within the purported membrane-spanning regions (20). There is also evidence that the actual ligand-binding region of the GTP-binding protein-linked receptors lies within these membrane-spanning regions (21). Thus, key amino acid changes in these regions could dramatically affect the ligand-binding properties.

Another possibility is a difference in the composition of the lipid bilayer in which the receptor protein is embedded. The lipid bilayer is believed to interact intimately with the membrane-spanning α-helical columns of the receptor protein. A different lipid environment could influence the tertiary structure of the α-helical columns. As stated earlier, ligand binding is believed to occur via multiple interactions within these membrane-spanning α-helical columns (21). Thus, ligand-binding properties could be affected by the lipid environment found in different species.

[2] K. C. Appell and J. Loscig, unpublished observations.

The results of this study offer a unique opportunity to investigate the nature of receptor-ligand interactions. The use of the antagonists in this study has demonstrated a species difference in the NK-1 receptor, despite the high level of sequence identity of the rat and human receptor proteins. In order to explain the species difference, two possibilities are raised, i.e., either key amino acid differences in the transmembrane binding region of the receptor protein or differences in the lipid environment in which the receptor protein is embedded. This study should provide the impetus for further research, such as site-directed mutagenesis in the receptor protein to test the protein hypothesis or expression of the rat and human receptor in the same cell line to test the lipid hypothesis. Furthermore, compounds such as the heterosteroids and their analogs should provide useful tools for discriminating between these hypotheses. Potentially, this would lead to a better understanding of ligand-NK-1 receptor interactions and progress towards the rational design of NK-1 receptor agonists and antagonists.

This study also emphasizes the importance of the choice of species in drug discovery and development strategies. Thus, the use of rats or mice to evaluate novel compounds at the NK-1 receptor would appear inappropriate if the compounds are targeted for therapeutic use in humans.

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References

1. Regoli, D., G. Drapeau, S. Dion, and R. Couture. New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. *Trends Pharmacol. Sci.* **9**:290–295 (1988).
2. Shigemoto, R., Y. Yokota, K. Tsuchida, and S. Nakanishi. Cloning and expression of rat neuromedin K receptor cDNA. *J. Biol. Chem.* **265**:623–628 (1990).
3. Van Giersbergen, P. L., S. A. Shatzer, A. K. Henderson, J. Lai, S. Nakanishi, H. I. Yamamura, and S. H. Buck. Characterization of a tachykinin peptide NK-2 receptor transfected in murine fibroblast B82 cells. *Proc. Natl. Acad. Sci. USA* **88**:1661–1665 (1991).
4. Dion, S., N. Rouissi, F. Nantel, D. Jukic, N. E. Rhaleb, C. Tousignant, S. Telemaque, G. Drapeau, D. Regoli, E. Naline, C. Advenier, P. Rovero, and C. A. Maggi. Structure-activity study of neurokinins: antagonists for the neurokinin-2 receptor. *Pharmacology* **41**:184–194 (1990).
5. Snider, R. M., J. W. Constantine, J. A. Lowe III, K. P. Longo, W. S. Lebel, H. A. Woody, S. E. Drozda, M. C. Desai, F. J. Vinick, R. W. Spencer, and H. Hess. A potent nonpeptide antagonist of the substance P (NK-1) receptor. *Science (Washington D. C.)* **251**:435–437 (1991).
6. Park, C. H., V. J. Massari, R. Quirion, Y. Tizabi, C. W. Shults, and T. L. O'Donohue. Characteristics of [^{3}H]-substance P binding sites in rat brain membranes. *Peptides* **5**:833–836 (1984).
7. Burcher, E., S. H. Buck, W. Lovenberg, and T. L. O'Donohue. Characterization and autoradiographic localization of multiple tachykinin binding sites in gastrointestinal tract and bladder. *J. Pharmacol. Exp. Ther.* **236**:819–831 (1986).
8. Lee, C. M., N. J. Campbell, B. J. Williams, and L. L. Iversen. Multiple tachykinin binding sites in peripheral tissues and in brain. *Eur. J. Pharmacol.* **130**:209–217 (1986).
9. Lee, C. M., W. Kum, C. S. Cockram, R. Teoh, and J. D. Young. Functional substance P receptors on a human astrocytoma cell line (U-373 MG). *Brain Res.* **488**:328–331 (1989).
10. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
11. Dahlquist, F. The meaning of Scatchard and Hill plots. *Methods Enzymol.* **48**:270–299 (1978).
12. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
13. Wang, C. S., and R. L. Smith. Lowry determination of protein in the presence of Triton X-100. *Anal. Biochem.* **63**:414–417 (1975).
14. Torrens, Y., J. C. Beujouan, M. Saffroy, M. C. Daguet de Montety, and L. Bergstrom. Substance P receptors in primary cultures of cortical astrocytes from the mouse. *Proc. Natl. Acad. Sci. U.S.A.* **83**:9216–9220 (1986).
15. Beajouan, J. C., Y. Torrens, A. Herbet, M. C. Daguet, J. Glowinski, and A.

Prochiantz. Specific binding of an immunoreactive and biologically active ^{125}I-labeled substance P derivative to mouse mesencephalic cells in primary culture. *Mol. Pharmacol.* **22**:48–55 (1982).
16. Burcher, E., and S. H. Buck. Multiple tackykinin binding sites in hamster, rat and guinea pig urinary bladder. *Eur. J. Pharmacol.* **128**:165–177 (1986).
17. Venepalli, B. R., L. D. Aimone, K. C. Appell, M. R. Bell, J. A. Dority, R. Goswami, P. L. Hall, K. B. Lawrence, M. E. Logan, V. Kumar, P. M. Scensny, J. A. Seelye, B. E. Tomczuk, and J. M. Yanni. Synthesis and substance-P receptor binding activity of androstano[2,3-*b*](pyrimido[1,2-*a*]benzimidazoles). *J. Med. Chem.* **35**:374–378 (1992).
18. Appell, K. C., B. E. Babb, R. Goswami, P. L. Hall, K. B. Lawrence, M. E. Logan, R. Przyklek-Elling, B. E. Tomczuk, and J. M. Yanni. Imidazo[4,5-*b*] quinoxaline cyanine dyes as substance P antagonists. *J. Med. Chem.* **34**:1751–1753 (1991).
19. Hershey, A. D., and J. E. Krause. Molecular characterization of a functional cDNA encoding the rat substance P receptor. *Science* (*Washington D. C.*) **247**:958–962 (1990).
20. Gerard, N. P., L. A. Garraway, R. L. Eddy, T. B. Shows, H. Iijima, J. Paquet, and C. Gerard. Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones. *Biochemistry* **30**:10640–10646 (1991).
21. Buck, S. H., R. M. Pruss, J. L. Kratensky, P. J. Robindson, and K. A. Stauderman. A tachykinin peptide receptor joins an elite club. *Trends Pharmacol. Sci.* **9**:3–5 (1988).

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