WIN 64821, A NOVEL NEUROKININ ANTAGONIST PRODUCED BY AN *Aspergillus* sp.

II. BIOLOGICAL ACTIVITY

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WIN 64821, a secondary metabolite produced by *Aspergillus* sp. (ATCC 74177) was found to inhibit radiolabeled substance P (SP) binding in a variety of tissues, including those of human origin. This compound inhibited, in a competitive manner, the binding of SP with *Ki* values ranging from 0.24 μM in human astrocytoma U-373 MG cells to 7.89 μM in rat submaxillary membranes. Additionally, WIN 64821 was found to inhibit ^{125}I-NKA binding to the NK2 receptor in human tissue at a concentration equivalent to its NK1 activity (0.26 μM). The inhibitory activity of WIN 64821 against an NK3 selective ligand, ^{3}H-senktide, was found to be much weaker ($Ki = 15.2\,\mu$M). WIN 64821 was also evaluated in NK1 functional assays and was found to be a competitive antagonist of SP-induced contractility in the guinea pig ileum ($pA_2 = 6.6$) as well as an inhibitor of SP-induced $^{45}Ca^{2+}$ efflux from human astrocytoma U-373 MG cells ($IC_{50} = 0.6\,\mu$M). In a rat vas deferens model, WIN 64821 inhibited eledoisin-induced contractility with an IC_{50} of 3.4 μM indicating functional antagonism at the NK2 receptor. The data presented in this study provide biochemical, pharmacological and functional evidence supporting WIN 64821 as a competitive neurokinin antagonist.

The tachykinins or neurokinins are a family of undecapeptides that share the common COOH-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2 (where X is Phe, Tyr, Val or Ile). Research has shown the existence of three distinct mammalian tachykinins termed substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Receptors for these ligands have been classed into three subtypes termed NK1, NK2 and NK3, respectively. SP, originally isolated from the brain and gut[1)] is the most intensively studied tachykinin and a putative neurotransmitter. SP, when released from the terminals of certain primary afferent neurons following nervous stimulus, can induce an excitatory effect on spinal dorsal horn neurons[2)] that generates a painful stimulus.[3)] In addition to a nociceptive role, SP has been shown to exist in elevated concentrations in nerves supplying sites of chronic inflammation[4)] and has been shown to cause histamine release from mast cells and basophils from different species.[5)]

In addition to the numerous studies pertaining to the NK1 receptor, the use of radioligands selective for either the NK2 or NK3 receptor have demonstrated the existence of these receptor subtypes in various tissue preparations. For example, using radioiodinated NKA, specific NK2 binding sites in rat duodenum (RD) have been identified.[6)] NK2 receptors have also been shown to exist in a number of other preparations including rabbit pulmonary artery[7)] and human urinary bladder.[8)] Additionally, the rat vas deferens has been shown to be a somewhat unique tissue in that, of the neurokinin class, it appears to possess solely NK2 receptors,[9)] making it a useful testing model for NK2 functional activity. While NK2 sites have been clearly demonstrated in peripheral tissues, their existence in the central nerveous system is somewhat

controversial.

NK3 receptor binding has been well characterized in brain preparations. Using ^{125}I-Bolton-Hunter-eledoisin, (BH-E, a radiolabeled tachykinin isolated from amphibians), the existence of NK3 receptors in rat brain membranes has been demonstrated.[10] Subsequent radioligand binding studies showed that BH-E and ^{3}H-Neurokinin B (NKB) labeled the same site in rat brain and exhibited identical pharmacological profiles and distribution.[11] NK3 receptor existence has been further confirmed by the highly selective agonist, senktide [succ-(Asp[6], MePhe[8])-SP(6-11)].[12] These investigators demonstrated that senktide was highly selective for NK3 receptors with little affinity for NK1 or NK2 receptors.

While many selective, peptide agonists for neurokinin receptors have been characterized,[7] very few peptide, *e.g.* (D-Pro[2], D-Trp[7,9])-SP[13] or nonpeptide, *e.g.* CP-96,345[14] and RP 67580[15] antagonists have been described.

Recent reports have detailed the presence of neurokinin-binding antagonists isolated from microbial sources. A cyclic depsipeptide with activity against both NK1 and NK2 receptors has been described.[16] In addition, nonpeptide inhibitors, termed fiscalins, with moderate NK1 binding activity have also been described.[17] There has also been a report of a tetracyclic compound, anthrotainin,[18] with NK1 activity; however, this compound was found to be a noncompetitive SP antagonist.

In the course of screening for inhibitors of SP binding to NK1 receptors we discovered a component present in a microbial extract which exhibited sub to low μM activity in a SP radioligand receptor binding assay. The compound, WIN 64821, is a secondary metabolite derived from an *Aspergillus* sp.[19] and consists structurally of a Trp-Phe diketopiperazine dimer.[20] We have found WIN 64821 to be unique as a natural product due to its potency and its nonpeptide structure.

This report describes the effects of WIN 64821 on mammalian neurokinin receptors, including those of human origin, and further defines its mechanism as a possible neurokinin antagonist. Additional studies on the fermentation, isolation and biosynthesis of WIN 64821 and analogs are reported in accompanying papers.[19,21]

Materials and Methods

Culture and Preparation of Human Astrocytoma Cells (HAC)

The culture medium consisted of Minimum Essential Medium (MEM) containing 10% heat inactivated horse serum, 2% MEM Amino Acid Solution, 2% MEM Vitamin Solution and 2% Penn-Strep solution. The final medium was prepared by filter sterilization through 500 ml Nalgene disposable (VWR). Human U-373 MG astrocytoma cells (ATCC No. HTB 17) were cultured in MEM replacing the medium twice weekly. Frozen cell stocks were prepared by centrifuging trypsonized cells in MEM ($250 \times g$ for 10 minutes), removing the medium, and adding medium containing 7.5% DMSO at 4°C (1.2 ml/75 cm^2 flask) to the pelleted cells. The cells (1.2 ml) were stored at −80°C and were rapidly thawed at 37°C prior to use. Sterile, Corning 96-well plates were pretreated at room temperature (RT) with 0.25 ml poly-D-lysine (50 μg/ml in phosphate buffered saline [PBS]) and incubated for 40 minutes at RT. The poly-D-lysine was then removed and each well was washed twice with 0.25 ml PBS. To each well was added 0.2 ml of thawed cells from above stock such that the final density was $1.4 \sim 1.9 \times 10^4$ cells/well. The culture was incubated for 2 days in an atmosphere of 95% air - 5% CO_2 and the resulting monolayer used for binding experiments.

^{125}I-BH-substance P Binding

The binding assay was performed on intact cells (90 ~ 100% confluent) in 96-well Corning plates. The medium was first removed and the cells were washed three times with 0.25 ml binding buffer (BB) at room temperature. The binding assay mixture (0.1 ml) consisted of 60 μl BB (which consisted of 25 mM HEPES

buffer, 5.8 mM KCl, 2.2 mM KH_2PO_4, 115 mM NaCl, 0.6 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1% bovine serum albumin (BSA), 5 mM glucose, 1 μM thiorphan, and 40 μg/ml bacitracin, final pH = 7.4), 0.1 nM ^{125}I-BH-SP (20 μl of 0.5 nM in BB), and 20 μl BB with test compound or 0.1 μM substance P (final concentration) to define nonspecific binding. The plates were incubated at 25°C for 90 minutes. The buffer containing unbound ^{125}I-BH-SP was removed and the cells were washed 4 × with 0.25 ml ice-cold BB. The cells were detached from the plates by the addition of 140 μl of 0.2% Triton X-100 in H_2O with 1 mg/ml BSA after which 100 μl was counted in a Packard Cobra gamma counter.

Preparation of Submaxillary Gland Membranes

Submaxillary glands from both sexes of Hartley guinea pigs (GPSM) or Sprague-Dawley rats (RSM) were received from Pel Freeze Biologicals (Rogers, Ark) *via* overnight express, in a plastic bag well-embedded in wet ice. The glands were separated from lymph, fat and other tissue and placed in ice cold buffer C at a ratio of 100 ml of buffer per 50 glands. The glands were then homogenized with a Brinkman Polytron 20TS generator using 3 or 4 ten-second bursts at a setting of 10. The resulting slurry was then centrifuged at 16,000 × g at 4°C for 15 minutes. The supernatant fluid was discarded and 200 ml of buffer B (consisting of 50 mM Tris buffer, 300 mM KCl and 10 mM EDTA, final pH = 7.40) was added to the pellet in order to release any native bound substance P from its receptor. The pellet was resuspended by homogenization in the Polytron at low speed. The suspension was allowed to incubate on ice for 30 minutes with periodic swirling. The mixture was centrifuged at 16,000 × g as before and the supernatant fluid discarded. Two-hundred ml of Tris buffer (50 mM, pH 7.40) was then added to the pellet; the pellet was resuspended and centrifuged as above. This process was repeated, after which a minimal amount of Tris buffer (about twice the pellet volume) was added to the membranes. The protein content was determined by the Bradford method (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard; the protein yield was typically 7 ~ 8 mg/ml, resulting in a total yield of approximately 250 mg/50 glands. The membrane preparations were stored in 1.0 to 1.5 ml volumes at −70°C.

Preparation of Brain Membranes

Fresh forebrain membranes were prepared from whole brain minus cerebellum from male, Sprague-Dawley rats (RFB), Hartley guinea pigs (GPB) or donated human fetal brain (HFB, International Institute for the Advancement of Medicine, Exton, PA) as described previously.[22] Briefly, the tissue was homogenized in 20 volumes of ice cold buffer C (which consisted of 120 mM NaCl, 5 mM KCl, and 50 mM Tris buffer, final pH = 7.40) with a Tissuemizer Mark II (8,500 rpm for 30 seconds). The homogenate was centrifuged at 48,000 × g for 10 minutes. The resulting pellet was resuspended using a motor-driven Teflon glass homogenizer in buffer B and incubated for 30 minutes at 4°C. The homogenate was then centrifuged, as above, twice in Tris buffer. The final pellet was resuspended in approximately 10 ml Tris buffer/2 ~ 3 g tissue wet weight for protein determination and storage as described above. When prepared in this manner, the protein yield for the GPB and RFB was typically 2.5 ~ 3.0 mg/ml (or about 30 mg membrane protein/2.5 g tissue wet weight).

Preparation of Human Urinary Bladder Membranes (HUB)

Frozen urinary bladder tissue (International Institute for the Advancement of Medicine, Exton, PA) was placed in a 2-liter plastic beaker containing minimal Buffer C and allowed to thaw at room temperature. Tissue that was obviously not detrusor muscle was trimmed. The bladder was cut open exposing the lumen and trimmed of obvious fat and connective tissue. The tissue was cut into 1 cm^2 cubes and placed in a small glass beaker with minimal buffer C. After mincing (by hand) the tissue was placed in an Erlenmeyer flask and brought to 10 volumes with buffer C. The tissue was homogenized for a 30-second burst at a setting of 6 with a Brinkman Polytron. The homogenate was poured into 250-ml conical bottles and spun at 1,500 × g for 10 minutes. The supernatant fluid was poured through two layers of cheese cloth into a beaker. Five volumes of buffer C were added to the pellet and rehomogenized as above. The homogenate was treated as above. The supernatant fluid from both preparations was combined and diluted with sufficient 10 × buffer B to yield a 1 × concentration and incubated for 30 minutes on ice. The mixture was then centrifuged for thirty minutes at 48,000 × g. The pellet was resuspended in Tris buffer with the Polytron. The suspension was recentrifuged at 48,000 × g for thirty minutes, the pellet washed once with

Tris buffer using the Polytron to resuspend the membranes, and recentrifuged as above. The final pellet was resuspended in Tris buffer containing 5 μg/ml chymostatin, 4 μg/ml leupeptin, 40 μg/ml bacitracin and 10 μM thiorphan using a 5-ml Potter homogenizer. The membranes were stored at -70°C at a concentration of 2.5 g wet weight/ml.

Neurokinin Receptor Binding Studies

For submaxillary NK1 binding, a membrane sample of either rat or guinea pig submaxillary membranes (RSM, GPSM, respectively) was thawed on ice and rehomogenized with a glass Dounce homogenizer. The test was performed in polystyrene test tubes containing a total volume of 0.5 ml as follows: 100 μl Tris buffer, 100 μl protease buffer (which contained 5 μg/ml chymostatin, 10 μg/ml leupeptin, 100 μg/ml bacitracin, 3 mM $MnCl_2$, 0.05% BSA, in 50 mM Tris buffer, pH 7.40 with or without unlabeled substance P), 100 μl ^{3}H-Substance P (approximately 0.75 nM) and 200 μl membrane preparation (containing 150 to 300 μg protein per tube). All tubes received 5 μl of DMSO or DMSO containing compound of interest, such that the 1 : 100 dilution yielded the desired concentration. The reaction was allowed to reach equilibrium (20 ~ 30 minutes) and then passed through Whatman GF/C filters using a Brandel harvester. The filters were washed three times each with 3 ml of ice-cold Tris buffer and counted in a Beckman LS5000 liquid scintillation counter. Nonspecific binding was determined in the presence of 1 μM unlabeled substance P and was generally less than 10% of the total. Studies on HFB (using 75 μg membrane protein/assay tube) and RFB (100 ~ 250 μg membrane protein/assay tube) NK1 binding utilized approximately 0.1 nM ^{125}I-Bolton-Hunter-Substance P as the ligand. Otherwise, the protocol was the same as that described for the submaxillary glands.

Calculations of NK1 Binding Parameters

Equilibrium dissociation constants (K_d) and receptor densities (B_{max}) were determined according to the classic method,[23] by Accufit Saturation-Two Site Analysis computer software (Beckman) using a non-linear curve fitting model.

Calculations of IC_{50} values (*i.e.*, the 50% inhibitory concentration) for experiments utilizing GPSM, RSM or HFB utilized a log-logit (L) transformation. For studies utilizing RFB as the membrane source, the competition curve data were analyzed by computer nonlinear least squares best fit of the data to the Hill equation[24] which determines the IC_{50} values and slopes (Hill coefficients) from at least 6 concentrations of the test compound.

Inhibitor affinity constants (*Ki*) were calculated as described previously.[25]

NK2 Receptor Binding

For NK2 binding experiments, the binding assay mixture (0.25 ml) contained 150 μl membrane protein (10 ~ 40 μg HUB), 0.2 nM ^{125}I-NKA (50 μl of 1 nM in Protease Buffer with 10 nM (Sar[9], Met(O_2)[11]) SP to block NK1 binding) and 50 μl of protease buffer containing test compound or 1 μM NKA (final concentration) to define nonspecific binding. The binding reaction was initiated by the addition of membranes. All assays were run in duplicate and the reaction mixtures were incubated for 90 minutes at 25°C. The assay mixtures were then diluted with 1 ml of Tris buffer containing 3 mM $MnCl_2$ (buffer D) and filtered through GF/C glass filters presoaked (at least three hours at room temperature) in 0.25% polyethylenimine, 0.3% Triton X-100, in Tris buffer. Filters were washed 7 times more with 1 ml buffer D. The radioactivity trapped on the filters was counted in a Packard Cobra Gamma Counter. The competition curve data were analyzed as described above for SP binding to RFB except $N = 7$ for these tests.

NK3 Receptor Binding

Fresh guinea pig brains were used for experiments investigating NK3 binding. Membranes were prepared as described for RFB or HFB. Assay tubes contained the same buffer concentrations and enzyme inhibitors as described for the NK1 assay using GPSM. The total volume in the assay tubes was 0.5 ml and ^{3}H-senktide was included at a concentration of 3.5 nM for the competition studies. Unlabeled senktide (10 μM) was used for the determination of nonspecific binding.

Guinea Pig Ileum Contractility Studies

Guinea pig ileal strips were prepared as previously described.[26] Briefly, male albino Charles River Hartley strain guinea pigs weighing between 300 and 600 g were used. The longitudinal muscle of the ileum was removed carefully from the circular muscle, segments of which were mounted into 10 ml baths at a resting tension of 1 g. Tissues were bathed with warm (31°C) oxygenated (95% O_2-5% CO_2) KREBS' solution (consisting of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, supplemented with atropine and pyrilamine at 1 μM concentrations) and were stimulated chemically with the spasmogen.

For all preparations, contractions were recorded isometrically with a Grass FT 03 C transducer on a polygraph recorder (Grass RPS 7c 8). After equilibration (60~90 minutes) solutions of compounds were added to the fluid bathing the tissue in 10 to 100 μl volumes.

Agonist concentration-effect curves were generated cumulatively, the preincubation time with an antagonist was 10 minutes and the washout time between agonist concentration-effect curves was a minimum of 60 minutes. In all experiments the EC_{50} (*i.e.*, the 50% effective concentration) for an agonist did not differ ($p < 0.05$) between consecutive concentration-effect curves in control preparations. Contraction height was expressed as a percentage of the maximum contraction height produced by the spasmogen before incubation of the test compound. For calculation of EC_{50}, regression analysis of the concentration-effect curves (percentage of effect *vs.* concentration) were performed using a log-probit analysis of the data.[27] Statistical differences between EC_{50} values and between ratios of EC_{50} values for different compounds post/pre incubation with a test agent were identified using a STUDENT's *t* test.

SP-induced ^{45}Ca Efflux

U-373 MG human astrocytoma cells were grown as described for use in NK1 binding. For efflux experiments, 3×10^5 cells were grown in 35 mm plates until confluency. The medium was changed every 2 days. The last change took place 1 day before the cells were used.

On the day of the experiment, the medium was removed by aspiration and replaced with 2 ml of efflux assay buffer (EAB; consisting of 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.4 mM Na_2HPO_4, 8.4 mM glucose, 20 mM HEPES buffer, 1 mM $CaCl_2$, and 0.5 μg/ml BSA) at room temperature containing 10~40 μCi of $^{45}CaCl_2$. The cells were incubated at 37°C with EAB containing $^{45}CaCl_2$ for 3~4 hours and then washed ten times with 2 ml of EAB to remove extracellular $^{45}Ca^{2+}$. All experiments were conducted at room temperature.

To determine the ability of compounds to inhibit substance P-induced $^{45}Ca^{2+}$ efflux, or to induce efflux, the sampling period was divided into two, 2-minute periods. Following the tenth 2 ml wash to remove excess $^{45}Ca^{2+}$ as described above, 1 ml of EAB or EAB containing antagonist was added to the plate. Two minutes later, 0.8 ml was transferred to a scintillation vial for counting and replaced by 0.8 ml of EAB containing substance P $\pm$ antagonist. Two minutes later 0.8 ml were removed for counting. After removal of the last sample, residual EAB was aspirated and the total remaining $^{45}Ca^{2+}$ was extracted twice with 1 ml of 2.0% sodium dodecyl sulfate (SDS) and counted by liquid scintillation spectroscopy.

^{45}Ca Fractional Release (FR) was defined as the amount of ^{45}Ca released during a 2 minute sampling period divided by the total amount of ^{45}Ca present in the cells at the beginning of that time period. Percent prestimulation FR, or % of control, was defined as the FR observed during the second 2-minute sampling period divided by the FR observed during the first 2-minute sampling (or control) period, multiplied by one hundred.

Data for % Inhibition curves were calculated after subtracting the efflux observed in the absence of substance P and antagonist, *i.e.*, in the presence of EAB only, during both sampling periods.

NK2 Functional Assay

The experimental preparation for the evaluation of the NK2 functional effects of WIN 64821 was similar to that described previously.[28] Briefly, male, Sprague-Dawley Taconic rats (approximately 200 g) were decapitated. Both vasa differentia were dissected free of connective tissue and tansferred to a petri dish containing KREBS' solution. Each organ was then stripped of its associated blood vessels and connective tissue sheath and semen was gently expressed from the lumen. Tissues were suspended individually under 1 g resting tension in 10 ml organ baths containing warmed (34°C), oxygenated (95% O_2-5% CO_2)

KREBS' solution.

Grass S-88 stimulators delivered repetitive field stimulation (0.1 Hz, 1 mseconds duration, single pulses) applied at the minimum voltage to produce a maximal contraction through platinum wire ring electrodes located at the top and bottom of each bath. Low frequency, transmural stimulation thus induced regular rapid contractions of the longitudinal muscle of the vas which were recorded *via* Grass FT.03 transducers on a Grass RPS 7D8 regulated power supply (model 7D). Tissues were equilibrated for 1 hour with stimulation. Bath fluid was replaced at 10~15 minutes intervals.

Eledoisin (1.0 μM) produced a potentiation of contractions which reached a stable, maximum height within 2~3 minutes. The effects of WIN 64821 and its vehicle equivalent on either basal or eledoisin-induced potentiation of contractions were determined by cumulative addition of test compound.

Either WIN 64821 or DMSO was administered in concentrations from 0.1 nM to 30 μM to both control preparations and those with 1 μM eledoisin. Data were expressed as % inhibition of either basal or eledoisin-stimulated (minus basal) contraction height. IC_{50} values were determined by regression analysis of the concentration-effect curves (percentage of effect *vs.* concentration) performed using a log-logit analysis of data. Statistical differences between IC_{50} values for different compounds post/pre incubation with a test agent were identified using the STUDENT's *t* test.

Reference Compounds and Chemicals

Triton X-100, HEPES, thiorphan, BSA, poly-D-lysine HBr (prepared as a 5 mg/ml 100 × stock in phosphate-buffered sarine (PBS)), and bacitracin were purchased from Sigma (St. Louis, MO). Salts and glucose were obtained from Eastman Kodak (Rochester, NY). KCl was purchased from Baker (J. T. Baker Inc. Phillipsburg, NJ), and all chemicals and components used in the HAC culture medium were obtained from Gibco (Life Technologies Inc., Gaithersburg, MD). ^{3}H-Senktide (83 Ci/mmol), ^{3}H-Substance P (44~54 Ci/mmol) and $^{45}CaCl_2$ (5 or 10 mCi (185 mBq) stock) were obtained from New England Nuclear; ^{125}I-Bolton-Hunter-Substance P (^{125}I-BH-SP) and ^{125}I-NKA (1,500~2,200 Ci/mmol) were obtained from Amersham, Inc (Arlington Heights, IL). Unlabeled senktide, substance P, and neurokinin A were obtained from Peninsula Labs (Belmont, CA). In the contractility experiments, pyrilamine, histamine diphosphate, and bradykinin acetate were purchased from Sigma. WIN 64821 was obtained from the Sterling Winthrop Natural Products Chemistry Department. All unlabeled drug dilutions were made in DMSO.

Results

Effects of WIN 64821 in NK1 Receptor Binding Assays

Since the activity of this compound was initially observed in a NK1 binding assay, considerable effort was devoted to characterization of this molecule as an inhibitor of SP binding. Early work using membranes from rat submaxillary glands and rat forebrains revealed *Ki* values (*vs.* SP) of 7.0 μM and 2.2 μM, respectively, for WIN 64821. We examined the compound in membrane preparations from other species and found similar or improved activity with greatest potency observed in HAC (Table 1).

The data in Table 1 indicate that WIN 64821 appeared to be 4-fold more potent in the human astrocytoma cell preparation than in human fetal brain. While we did see some evidence of tissue specificity for the compound (*cf.* rat brain *vs.* rat submaxillary gland in Table 1), the apparent greater potency in the HAC preparation was somewhat unique. When testing WIN 64821 in, for example, a guinea pig submaxillary membrane preparation, we observed a plateau in the concentration-response curve above 10 μM. This was in contrast to experiments with HAC where a full response was observed (Fig. 1). These data indicated that the HAC medium might allow for greater solubility of WIN 64821 or exhibit some other property which contributed to a shift in its binding potency. Examination of the binding properties of WIN 64821 in a GPSM preparation using either Tris buffer or HAC tissue culture medium resulted in a shift in the K_i

Table 1. The biological activity of WIN 64821 in various neurokinin binding assays.

Receptor	Source	Ligand	$K_i \pm$SEM (N)	Hill[a]
NK1	HFB	^{125}I-SP	1.01+0.27 (2)	1.1
	HAC	^{125}I-SP	0.24+0.10 (7)	1.0
	GPSM	^{3}H-SP	1.25+0.24 (9)	0.9
	GPB	^{3}H-SP	1.67+0.06 (2)	0.6
	RSM	^{3}H-SP	7.89 (1)	0.8
NK2	HUB	^{125}I-NKA	0.60+0.80 (3)	0.9
	RD	^{125}I-NKA	0.26 (1)	NC[b]
NK3	GPB	^{3}H-Senktide	15.2+4.7 (2)	0.5

[a] Hill coefficients calculated according to Materials and Methods.
[b] No calculation possible due to missing data.

Fig. 2. Scatchard plots representing the inhibition of specific ^{3}H-SP binding by three concentrations of WIN 64821 using GPSM membranes.

● 1.8 μM, ▲ 0.6 μM, ◆ 0.2 μM, ■ no inhibitor.

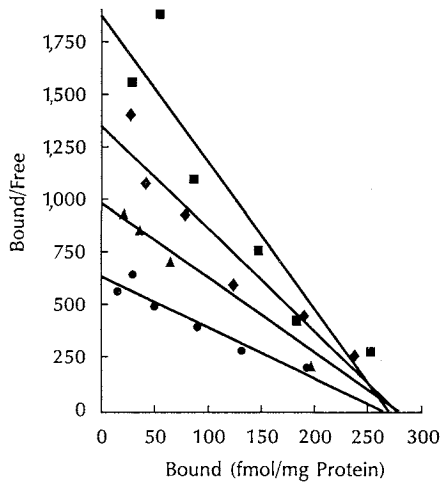


Fig. 1. Comparison of concentration-response curves of WIN 64821 with human astrocytoma cells (HAC, ◆) and guinea pig submaxillary membrane (GPSM, ■) NK1 preparations.

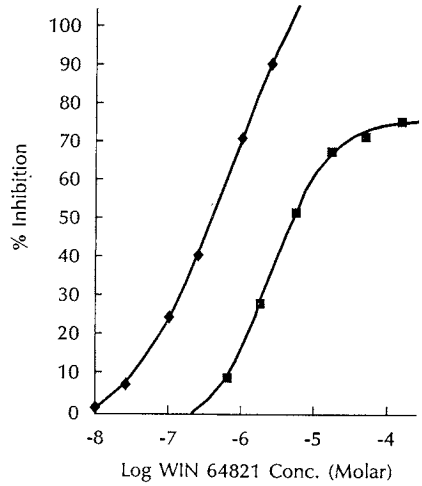


Table 2. Scatchard analyses of SP binding to guinea pig submaxillary membranes in the presence of WIN 64821.

WIN 64821 (μM)	SP K_d (nM)	B_{max} (fmole/mg)
0	0.15	273
0.2	0.22	290
0.6	0.27	279
1.8	0.46	281

from 1.4 μM in Tris buffer to 0.6 μM in the astrocytoma medium. In an additional experiment (data not shown) to ascertain the component(s) in the HAC medium responsible for this phenomenon, we concluded that the presence of BSA at 1% was the most significant factor in causing this effect.

WIN 64821 Competition Studies

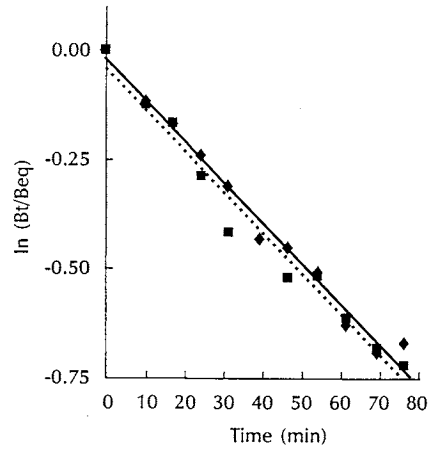
To determine the nature of the interaction between WIN 64821 and the NK1 receptor, we conducted experiments to examine whether this compound was acting competitively to inhibit SP binding at this site. Two experiments were performed to assess this property. In the first test, performed with GPSM, WIN 64821 was incubated at several concentrations in normal saturation binding experiments using ^{3}H-SP as the radioligand (0.03~1.0 nM). Scatchard analyses on the individual binding curves were performed at each concentration of WIN 64821 (Fig. 2). An analysis of the binding parameters of SP at each of the concentrations of WIN 64821 indicated that the WIN compound was acting in a competitive manner to inhibit SP binding. The shift in apparent K_d for SP with no change to the B_{max} values was indicative of a competitive interaction by WIN 64821 at the SP binding site (Table 2).

A second experiment examined the kinetics of SP binding to HAC by measuring the dissociation rate of ^{125}I-SP in the presence and absence of WIN 64821. In these experiments, cells were preincubated

90 minutes in the presence of 0.1 nM ^{125}I-SP to allow binding to reach equilibrium. At each of the indicated time points 0.1 μM unlabeled SP in the presence or absence of 7.5 μM WIN 64821 was added to the reaction mixture. Data generated from these experiments were linearized according to the equation of first-order kinetics, yielding a straight line with a slope or dissociation rate constant for binding conducted in the presence or absence of WIN 64821. As shown in Fig. 3, the presence of WIN 64821 had no effect on the rate of dissociation of ^{125}I-SP ($K_{off}=0.0098$ minute^{-1}; N=2) when compared with the dissociation rate in the absence of test compound ($K_{off}=0.00995$; minute^{-1}; N=2). The lack of an effect of WIN 64821 on the rate of dissociation of ^{125}I-SP is also consistent with a competitive interaction of this compound with the NK1 receptor.

Fig. 3. The effect of WIN 64821 on the dissociation rate of ^{125}I-SP from HAC.

■ Control, ◆ WIN 64821.



Each point is the mean from two experiments.

Effect of WIN 64821 on NK2 and NK3 Receptor Binding

While initial observations with WIN 64821 revealed activity at the NK1 receptor, we conducted additional tests to determine the binding profile of this compound at the other known neurokinin receptors. Experiments were conducted with HUB membranes to examine the activity of WIN 64821 at the NK2 receptor. This preparation contained NK2 receptors with affinity for ^{125}I-NKA similar to reported values.[8] WIN 64821 was found to exhibit excellent activity in this preparation ($Ki=0.60$ μM, N=4).

Using ^{3}H-senktide as the radioligand, experiments were designed to examine the activity of WIN 64821 at the NK3 receptor in guinea pig forebrain. Results from these experiments revealed much weaker activity with a *Ki* for WIN 64821 determined to be 15.2 μM (N=2).

These data revealed that WIN 64821 was equipotent against binding of ligand to the NK1 and NK2 receptors; however, the compound was approximately 20-fold less active at the NK3 site (Table 1).

Effect of WIN 64821 Guinea Pig Ileum Contractility

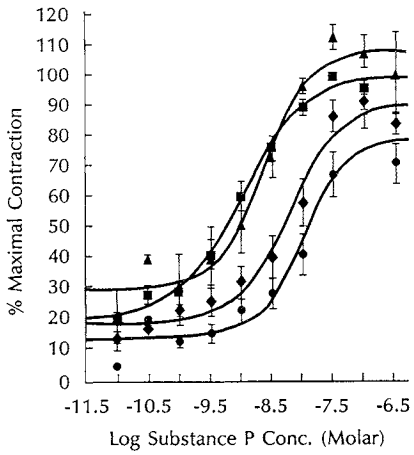
In order to confirm functional activity for WIN 64821 in SP mediated biological events, the compound was tested in a guinea pig ileum contractility model. In these experiments WIN 64821 was tested at three concentrations (1, 3 and 10 μM) and examined for its ability to inhibit SP-induced contractions. WIN 64821 produced rightward parallel shifts in the concentration effect curve for SP (Fig. 4) indicating that the compound was acting as a competitive antagonist of SP. We did notice, however, that the maximal effect of SP was reduced about 15% at 10 μM WIN 64821 indicating possible toxicity problems at the high concentration. A schild analysis of the data[29] yielded a slope of -0.75 ± 0.19 and a Pa_2 value of 6.6 ± 0.31 (Fig. 5). When the slope of the Schild plot was constrained to -1.0, the pA_2 was 6.2 translating to an EC_{50} of 0.63 μM. This value agrees well with the *Ki* values obtained from the binding studies.

Effect of WIN 64821 on ^{45}Ca Release

In an additional study examining the activity of WIN 64821 as a functional antagonist for SP, the

Fig. 4. The effect of WIN 64821 on SP-induced contractions in isolated guinea pig ileum.

● 10.0 μM, ◆ 3.0 μM, ▲ 1.0 μM, ■ control (no WIN 64821).

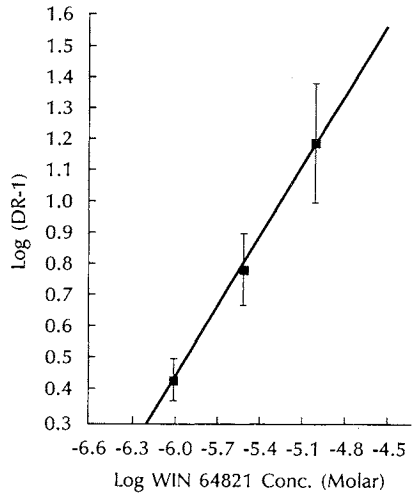


Each data point represents the mean ± SEM from at least three experiments. Data from each curve were fit to a 4-parameter, nonlinear equation at follows:

$$f_{(x)} = A + \frac{B-A}{1+(10^c/10^x)^D}$$

where X is the logarithm of concentration at the middle of the curve (log[IC_{50}]), A is the bottom plateau of the curve (usually 0), B is the top plateau of the curve (usually 100), and D is the Hill coefficient (usually set to 1).

Fig. 5. Schild plot of the antagonism by WIN 64821 of contractions elicited by SP in guinea pig ileum (data from Fig. 4).



DR is the dose ratio of SP that elicited the same 50% response in the presence and absence of the various concentrations of WIN 64821. Constraining the slope of the line to −1.0 resulted in a pA_2 of 6.2 or an EC_{50} of 0.63 μM.

Table 3. Effect of WIN 64821 on $^{45}Ca^{2+}$ efflux in human astrocytoma cells.

Stimulus	$^{45}Ca^{2+}$ release[a]
Test 1	
Buffer	46
SP (10 nM)	271
WIN 64821 (0.5 μM)	20
Test 2	
Buffer	−35
SP (3 nM)	143
WIN 64821 (10 μM)	−29

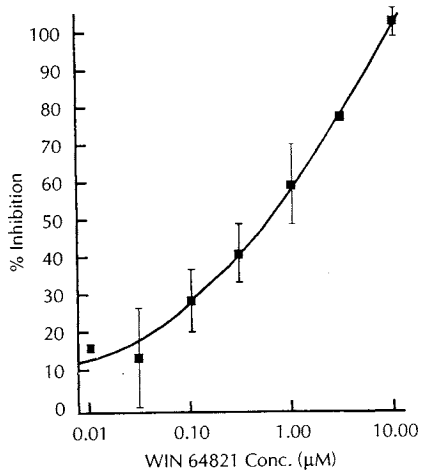
[a] Measured as % increase from T_0.

compound was tested for its ability to inhibit SP induced Ca^{2+} release from astrocytoma cells. SP itself produced, on average, a two and one-half to five fold increase in $^{45}Ca^{2+}$ efflux from U-373 astrocytoma cells. The EC_{50} for SP was calculated to be 1.2 +/− 0.7 nM. When WIN 64821 was tested for its ability to induce $^{45}Ca^{2+}$ efflux, itself, no effect was observed (Table 3). The compound was effective, however, as an inhibitor of $^{45}Ca^{2+}$ efflux induced by 3 nM SP (Fig. 6). The mean IC_{50} for 3 experiments was 0.6 ± 0.3 μM.

Effect of WIN 64821 in a NK2 Functional Model

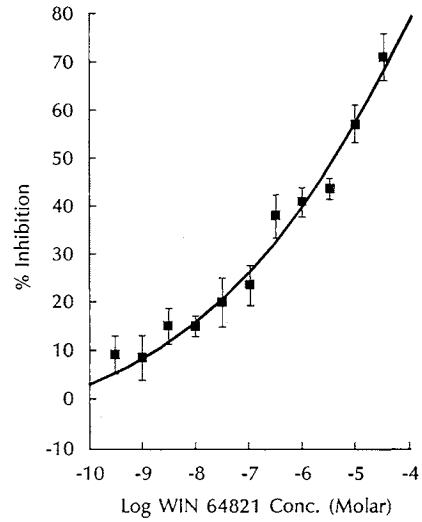
In the NK2 rat vas deferens assay, WIN 64821 inhibited the eledoisin-induced potentiation of twitch in a concentration dependent manner, (Fig. 7). The IC_{50} of WIN 64821 in this preparation was calculated to be 3.4 +/− 1.3 μM. On preparations not treated with eledoisin, WIN 64821 had no significant effect on electrically stimulated twitch height in concentrations up to 30 μM. While the DMSO did not significantly attenuate basal contractions, the vehicle alone did produce a concentration-dependent and significant ($p < 0.05$) inhibition of eledoisin-potentiated twitch height up to 45.5% at a level of vehicle equivalent to that used for 30 μM WIN 64821. Although the DMSO vehicle alone produced some inhibition of which

Fig. 6. Effect of WIN 64821 on SP-induced $^{45}Ca^{2+}$ efflux from HAC.



Each data point represents the mean±SEM from at least three experiments.

Fig. 7. Effect of WIN 64821 on eledoisin-induced contractions in electrically stimulated rat vas deferens.



Each data point represents the mean±SEM from at least three experiments.

height, the magnitude of the inhibition by WIN 64821 was significantly greater ($p<0.05$).

Discussion

The antagonism of postsynaptic neurokinin binding is a potentially novel approach toward the discovery of antinociceptive and anti-inflammatory agents. Information regarding the structure of neurokinin receptors is very scarce and, due to the lack of nonpeptide antagonists, little is known about either the active site of the receptor(s) or the pharmacophore of known neurokinin-active compounds. *De novo* synthesis of potentially active compounds is therefore impossible at this time. As such, in addition to chemical file screening, natural products were also screened for the presence of agents showing activity at this site.

To our knowledge, WIN 64821 is the first nonpeptide, competitive SP inhibitor discovered from natural sources, in this case from a fungal extract. The results from the neurokinin receptor binding studies also demonstrated equipotent activity at both the NK1 and NK2 receptors. Other nonpeptide inhibitors have been recently described; most notably CP-96,345, a potent quinuclidine SP antagonist[14] and RP 67580, an isoindole antagonist with nM potency;[15] however, these compounds are all selective NK1 antagonists.

Although many pharmacological preparations have been reported to elicit NKB-induced functional activity, the actual specificity of such activity remains suspect due to a large amount of receptor cross reactivity.[30,31] The lack of specific NK3 functional assays, coupled with the low affinity of WIN 64821 at this receptor argued against attempts to identify specific NK3 functional activity by WIN 64821. Further studies could be warranted if analogues of WIN 64821 are found with greater potency at this site.

In related experiments, WIN 64821 was examined in a number of other receptor targets in order to profile more completely the binding activity of this compound (data not shown). These included bradykinin B_2, muscarinic (M_1, M_2, and M_3), nitrendipine, angiotensin, atrial natriuretic factor, cholecystokinin (a and b), insulin, neuropeptide Y, thyrotropin releasing hormone, vasopressin, vasoactive intestinal polypeptide, Na channel (site III), and corticotropin releasing factor. WIN 64821 was tested at 10 μM in all of these preparations and was found to be inactive in all except the cholecystokinin b assay where a *Ki* of 1.4 μM was determined. This finding is interesting from the standpoint of possibly defining a common

pharmacophore for these three receptors (*i.e.*, NK1, NK2 and cholecystokinin b). This phenomenon warrants further investigation.

The data from the present study indicate that WIN 64821 interacted both biochemically and functionally, with approximate equal affinity at both NK1 and NK2 neurokinin receptors. The activity of this compound in NK2 binding and functional experiments was unexpected. However, such an action could prove efficacious in pain models since recent reports have provided evidence for the involvement of both NK1 and NK2 receptors in the modulation of thermal nociceptive processes.[32,33] It is conceivable that a compound with mixed (NK1 and NK2) antagonist activity could exhibit potent antinociceptive activity, possibly greater than a pure NK1 antagonist.

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