The Journal of Pharmacology and Experimental Therapeutics

Pharmacological Characterization of ZD6021: A Novel, Orally Active Antagonist of the Tachykinin Receptors

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Received November 17, 2000; accepted April 9, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The tachykinins, substance P, neurokinin A, and neurokinin B, have been implicated in many diseases. The present study evaluated the pharmacological properties of a novel tachykinin antagonist ZD6021 [3-cyano-N-(2S)-2-(3,4-dichlorophenyl)-4-[4-[(2-(methyl(S)-sulfinyl)phenyl)piperidino]butyl]-N-methyl-napthamide]. The affinity (K) of ZD6021 for the cloned human neurokinin (NK)1, NK2, and NK3 receptors was 0.12 ± 0.01, 0.64 ± 0.08, and 74 ± 13 nM, respectively. Mucin secretion by Chinese hamster ovary cells transfected with the human NK1 receptor was dose dependently inhibited by ZD6021: pIC50 = 7.6 ± 0.1. For NK1 and NK2 receptors, the agonist concentration-response curves using isolated tissues were displaced rightward in the presence of ZD6021: rabbit pulmonary artery, pA2 = 8.7 and 8.5; human pulmonary artery and bronchus, pKb = 8.9 ± 0.4 and 7.5 ± 0.2, at 10⁻¹ M, respectively. Serktide-induced contractions of isolated guinea pig ileum were also blocked by low concentrations of ZD6021. Oral administration of ZD6021 to guinea pigs dose dependently attenuated tracheal extravasation of plasma proteins induced by the NK1 receptor agonist Ac-[Arg6,Sar9,Met(O2)11]-SP(6-11), ED50 = 0.8 μmol/kg, and bronchoconstriction elicited by the NK2 receptor agonist [β-Ala6]-NKA(4-10), ED50 = 20 μmol/kg. Potency was unaffected by feeding. After oral administration of ZD6021, the time to peak activity was 150 min for the NK1 receptor and 60 min for the NK2 receptor with pharmacodynamic half-lives of 280 and 458 min, respectively. These data indicate that ZD6021 is a potent, orally active antagonist of all three tachykinin receptors. This compound may be useful for future studies of tachykinin-related pathology such as asthma.

The structurally related tachykinins (or neurokinins), substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), are widely distributed in the central and peripheral nervous systems. Biological effects of these neuropeptides are carried out via binding to their preferred receptors, NK1, NK2, and NK3, respectively, which are members of the G protein-coupled receptor superfamily. Activation of the tachykinin receptors influences a broad array of biological actions, including contraction, secretion, immune responses, and neurotransmission. The functions of a number of tissues (cardiovascular, respiratory, digestive, reproductive, excretory, and musculoskeletal) are influenced by these neuropeptides (Otsuka and Yoshioka, 1993).

SP and NKA are encoded from a single gene (preprotachykinin A or PPT-A), which gives rise to multiple mRNAs, (α, β, and γ) through RNA splicing (Krause et al., 1987). The amounts of these precursors are regulated in a tissue-specific manner; this determines, in part, the local levels of the neuropeptides (Otsuka and Yoshioka, 1993). Coexpression of SP and NKA exists in many tissues: human skin (Schulze et al., 1997), human bladder (Smet et al., 1997), rodent tendons, and joint capsule (Ackermann et al., 1999) and guinea pig airways (Kummer et al., 1992). The NKB gene (PPT-B) shows structural similarity to PPT-A; however, the preprotachyklin A and B mRNAs differ in the major sites of their expression (Kotani et al., 1986). Recently, SP and NKB were both detected in rodent ileum (Yunker et al., 1999). Up-regulation of the PPT genes and mRNAs for the neurokinin receptors occurs both in animal models of disease, such as allergic inflammation of the lungs (Fischer et al., 1996) and in human diseases, such as asthma (Adcock et al., 1993; Bai et al., 1995).

Development of structurally diverse tachykinin receptor-selective antagonists has advanced our understanding of the biological actions of these neuropeptides. Clinical studies have shown that these antagonists (particularly NK1 receptor antagonists) offer potential management of emesis (Na-vari et al., 1999) and other illnesses such as major depression (Kramer et al., 1998). However, NKA and NKB also bind

ABBREVIATIONS: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; PPT, preprotachykinin; ZD6021, 3-cyano-N-(2S)-2-(3,4-dichlorophenyl)-4-[4-[(2-(methyl(S)-sulfinyl)phenyl)piperidino]butyl]-N-methyl-napthamide; ASM-SP, Ac-[Arg6,Sar9,Met(O2)11]-substance P; ASM, Ac-[Arg6,Sar9,Met(O2)11]-peptide induced by the NK1 receptor agonist Ac-[Arg6,Sar9,Met(O2)11]-SP(6-11); ED50 = 0.8 μmol/kg, and bronchoconstriction elicited by the NK2 receptor agonist [β-Ala6]-NKA(4-10), ED50 = 20 μmol/kg. Potency was unaffected by feeding. After oral administration of ZD6021, the time to peak activity was 150 min for the NK1 receptor and 60 min for the NK2 receptor with pharmacodynamic half-lives of 280 and 458 min, respectively. These data indicate that ZD6021 is a potent, orally active antagonist of all three tachykinin receptors. This compound may be useful for future studies of tachykinin-related pathology such as asthma.

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with moderate affinity to the NK1 receptor (Maggi and Schwartz, 1997), possibly at a site distinct from the SP binding domain (Wijkhuisen et al., 1999). This nonselective nature of tachykinin binding, as well as the colocalization of these neuropeptides within tissue, suggests that some diseases, in particular asthma, might benefit from blockade of more than one tachykinin receptor. For example, Turner et al. (1996) have reported that airway hyper-reactivity and inflammation in nonhuman primates were synergistically improved by treatment with both NK1 and NK2 receptor antagonists compared with separate treatment with either compound alone. Thus, we have developed a series of compounds with affinity for all of the three tachykinin receptors.

This report provides the pharmacological characterization of a series example, 3-cyano-N-(25)-2-(3,4-dichlorophenyl)-4-[4-(2-(methyl-(S)-sulfinyl)phenyl)piperidino]butyl)-N-methyl-naphthamide (ZD6021). The data indicate that ZD6021 is orally available and is a potent antagonist of all three tachykinin receptors. Future studies of tachykinin-related pathology such as asthma may benefit from this pharmacological tool.

**Experimental Procedures**

**Binding Studies.** The cloning, heterologous expression and scale-up growth of MEL cells transfected with either the NK1, NK2, or NK3 receptor were conducted as previously published for the human NK1 receptor (Graham et al., 1991; Takeda et al., 1991; Huang et al., 1992; Aharony et al., 1994). The human NK1 receptor was identical to that reported previously (Gerard et al., 1991; Fong et al., 1992), whereas the human NK2 receptor differed from the genomic sequence at AA439 (Cys versus Phe; Buell et al., 1992; Takahashi et al., 1992).

Membranes from recombinant MEL cells were prepared as previously described (Aharony et al., 1994). Cells were homogenized at 4°C (Brinkman PT-20 polytron, Westbury, NY) in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 5 mM KCl, 120 mM NaCl, 10 mM MgCl2, 1 mM MnCl2, 25 mM NaHCO3, and 11 mM glucose. The buffer (Filtermate 196; Packard Instrument Co., Meriden, CT; and Modular Instruments, Malvern, PA) were sacrificed by exposure to CO2. A 15- to 20-cm small intestine, male Hartley guinea pigs (150–300 g; Hilltop Labs, Scottdale, PA) were sacrificed with 10% trichloroacetic acid:1% phosphotungstic acid. The precipitated glycoconjugates were counted (Packard Tri-Carb 2200CA, Downers Grove, IL). A secretory index was calculated by dividing the counts from the stimulation period by the counts from the previous equilibration period. Triplicate measurements were obtained for at least three experiments to derive an average secretory index. The IC50 was determined by curve-fitting the mean data in a nonlinear regression analysis using a sigmoidal dose-response with a variable slope and expressed as the log IC50 (pIC50) value.

**Isolated Tissue Responses.** Male New Zealand White rabbits (2–3 kg) were administered heparin (2.5 U/kg) and sodium pentobarbital (60 mg/kg i.v.). A bilateral thoracotomy was performed and the left and right branches of the pulmonary artery were excised, trimmed free of connective tissue, and cut into 5-mm rings. In some cases, the endothelium was removed by gentle abrasion of the intimal surface. The segments were suspended in water-jacketed tissue baths, thermostatted at 37°C and containing physiological salt solution comprised of 119 mM NaCl, 4.6 mM KCl, 1.8 mM CaCl2, 0.5 mM MgCl2, 1 mM NaH2PO4, 25 mM NaHCO3 and 11 mM glucose. The medium was gassed continuously with O2:CO2 (95:5). Initial tension was set at 2 g and stabilized for 30 min. Changes in tension were monitored by force transducers (Grass FT-03, Quincy, MA) coupled to a data acquisition system (Grass polygraph, model 7, interfaced with MF4 Modular Instruments, Malvern, PA).

Changes of NK1 or NK2 receptor-mediated vessel relaxation or contraction (minus endothelium) were stimulated either with cumulative additions of ASM-SP or the NK2 receptor agonist b-alanyl-NKA(4-10) (BANK), respectively. Propanolol (1 μM) and thiorphan (1 μM) were first added to the bath medium. For NK1 receptor activity, the tissue was contracted with phenylephrine (1 μM), stabilized, and then the selective NK1 receptor antagonist ZM274773 (30 nM, H. G. Bartholow and W. L. Ramsey, unpublished observation) was added to prevent potential NK1 receptor activation by the agonist. ASM-SP-induced relaxation was normalized to the maximum change produced by papaverine (1 mM). For NK2 receptor mediated contraction, tissue viability was first evaluated using KCl (30 mM) and the responses to BANK referenced to the maximal change stimulated by BaCl2 (30 mM). Concentration-response effects of ASM-SP or BANK were obtained in the absence or presence of ZD6021 (paired comparisons), which was incubated with the tissues for 90 or 30 min, respectively.

For measurement of NK1 receptor-mediated contraction of the small intestine, male Hartley guinea pigs (150–300 g; Hilltop Labs, Scottsdale, PA) were sacrificed by exposure to CO2. A 15- to 20-cm length of the ileum was removed at the junction of the cecum and placed in Krebs-Henseleit buffer containing 3 mM indomethacin. The lumen of the ileum was rinsed with buffer and sectioned into 15-mm segments. Each segment was fitted over a borosilicate glass Pasteur pipette and gently sliced longitudinally to cut through only the outer, longitudinal layer of muscle. This tissue was then separated from the inner, circular layer using a cotton-tipped swab. The longitudinal segments were suspended in tissue baths containing Krebs-Henseleit medium gassed with O2:CO2 (95:5) and thermostatted at 37°C. Initial tension was set at 1 g and the tissues were equilibrated for 60 min prior to addition of ZD6021. After a 2-h
exposure to ZD6021, thiorphan (1 mM) was added to each bath, and 15 min later, the dose-response to senktide was begun. Responses were referenced to a maximal contraction elicited by 1 mM BaCl₂.

In separate experiments, it was noted that the contraction to all concentrations of senktide was abolished by atropine (1 μM), indicating the cholinergic nature of this response. Activation of NK₃ receptors by either NKB or senktide has been shown to release acetylcholine in the small intestine via voltage-sensitive calcium channels (Yau et al., 1992). There was no effect of ZD6021 (1 μM) on cholinergic contraction evoked by electrical field stimulation.

Normal human lung tissue was obtained from anonymous donors (supplied by the International Institute for the Advancement of Medicine, Exton, PA, or the Anatomical Gift Foundation, Woodbine, GA). The tissue was generally obtained from motor vehicle accident victims. It was shipped in ice-cold RPMI-1640 medium and received for experimentation within 24 h of organ removal. Upon delivery to the laboratory, the tissue was immediately transferred to Krebs’ bicarbonate buffer and evaluated as described previously (Pedersen et al., 2000). Rings (8–10 mm in length) of pulmonary artery (NK₁) or human bronchus (NK₂) were suspended in tissue baths maintained at 37°C. Experimental conditions, including ancillary pharmacological additions, were similar to those described above with the exception that SP and NKA served as the agonists and the selective NK₂ receptor antagonist was omitted from the bath medium. Exposure of the tissue to ZD6021 for a period of 1 and 2 h (NK₁ and NK₂ receptors, respectively) afforded a maximal effect at each dose of the antagonist.

Agonist EC₅₀ values were determined by linear regression analysis and expressed as the negative logarithm (−log molar EC₅₀). The EC₅₀ values for all agonists were calculated at 50% of the maximum response produced by each agonist. Apparent Kᵦ values were calculated using the standard equation Kᵦ = (agonist/dose ratio – 1), where dose ratio was equal to antilog (−(−log molar EC₅₀ without antagonist) – (−log molar EC₅₀ with antagonist)). The resulting Kᵦ values were expressed as −log molar Kᵦ. Schild analysis was carried out (GraphPad Software) using multiple concentrations of ZD6021.

Tracheal Extravasation. A modified method of Saria et al. (1983) was used with Evans blue dye as an indicator of protein extravasation. Male guinea pigs (250–400 g) were anesthetized (1.5 g/kg i.p.) and surgically implanted with a jugular catheter for administration of compounds (jugular vein) and recording of blood pressure (carotid artery). The trachea was cannulated and connected to a heated pneumotachograph (Fleisch 0000; Switzerland), which was also attached to a Validyne differential pressure transducer (model MP45-14). Transpulmonary pressure was obtained by measuring the pressure difference between an intrapleural cannula placed in the 5th intercostal space and a side-arm adapter of the tracheal cannula with a second Validyne differential pressure transducer (model 45-24). Pulmonary resistance (Rₚ) and dynamic lung compliance were calculated (Modular Instruments). The animals were pretreated intravenously with ancillary pharmacophores (indomethacin, propranolol, and thiorphan) as described above.

The dose-response effects of cumulative intravenous injections (at 10-min intervals) of either the selective NK₁ or NK₂ receptor agonists (ASM-SP or BANK) were obtained by measuring the peak airway response. In some cases, capsaicin was given as the agonist to release endogenous tachykinins (Saria et al., 1985). Baseline resistance was referenced as 100% conductance [airway conductance (Gₚ)] or the reciprocal of pulmonary resistance (1/Rₚ). The peak value of Gₚ following agonist administration was expressed as a percentage of the baseline value obtained before agonist was added. The ED₅₀ value was calculated as the agonist dose resulting in a reduction of Gₚ to 50% of baseline. All ED₅₀ values were converted to the negative logarithm and expressed as −log ED₅₀ for statistical analyses. Only one agonist dose-response curve was obtained in each animal. The “agonist” ED₅₀ values obtained in the presence (P) and absence (A) of antagonist were used to calculate a dose ratio (P/A) and this value was used as an expression of potency of ZD6021.

Potency of ZD6021 was also presented as an ED₅₀ value and as a percentage of protection in time-response studies. Using the experimental protocol described above, the dose of ZD6021 that resulted in only a 50% decrease in conductance after receiving 0.1 nmol/kg i.v. BANK was defined as the “agonist” ED₅₀ (more commonly referred to as an ID₅₀). Protection by ZD6021 was expressed as a percentage of the difference between the conductance obtained from the ZD6021-treated group and that found for the control animals divided by the difference between the maximal conductance value and the control group conductance value.

Statistical Analyses. Data were expressed as mean ± S.E.M. Statistical differences were determined using ANOVA/Tukey-Kramer or Student’s t test, with a minimum significance of p < 0.05.

Chemicals. The tachykinin antagonist ZD6021 (Fig. 1) was prepared as the crystalline citrate salt. This compound and other tachykinin antagonists (SR 140333, ZD7944, ZM247733) were synthesized in AstraZeneca chemistry research laboratories. For in vitro experiments, ZD6021 was dissolved in dimethyl sulfoxide. For in vivo work, it was dissolved in 30% polyethylene glycol 400 balanced in saline. Thiorphan, indomethacin, and propranolol were purchased from Sigma (St. Louis, MO). Senktide was obtained from Penninsula Laboratories (San Carlos, CA) and ASM-SP and BANK from Cambridge Research Laboratories (Cheshire, UK).

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Results

In Vitro Pharmacology. Figure 2 shows that affinity of ZD6021 for the human tachykinin receptors was high. The binding of either [3H]SP or [3H]NKA to its putative receptor was potently inhibited by ZD6021: $K_i = 0.12 \pm 0.1 \text{nM} (n = 10, pK_i = 9.95 \pm 0.04)$ and $0.61 \pm 0.7 \text{nM} (n = 6, pK_i = 9.23 \pm 0.05)$, respectively. Comparatively, ZD6021 inhibition of ligand ([125I-MePhe7NKB] binding at the NK$_3$ receptor was markedly lower: $K_i = 74.0 \pm 0.7 \text{nM} (n = 3, pK_i = 7.13 \pm 0.04)$. Binding affinity of ZD6021 for nontachykinin receptors (or sites) with $K_i$ values less than 1 $\mu$M was limited to the dopamine D$_{3RT}$ receptor (90 nM), the muscarinic M$_3$ (509 nM) receptor, the dihydropyridine L-type calcium channel (606 nM), and the sigma nonselective site (777 nM). Although binding affinity at the muscarinic M$_3$ receptor was low ($K_i = 1.2 \mu$M), it was the only nontachykinin site that showed marked antagonism (>50%) of receptor activation by ZD6021 (10 $\mu$M) in an isolated tissue assay (guinea pig ileum).

Activation of the NK$_1$ receptor results in epithelial cell secretion of mucus. Excessive secretion is a component of inflammatory diseases such as asthma. We therefore examined the ability of ZD6021 to prevent mucin secretion. Stimulation of Chinese hamster ovary cells transfected with the human NK$_1$ receptor with 10 $\mu$M ASP-SP induced maximal mucin secretion; a 175 $\pm$ 6% increase in the secretory index over basal values. The sensitivity, expressed as a $pD_2$ value, of these cells for ASP-SP was 8.91 $\pm$ 0.13. The increased mucin secretion caused by 100 nM ASP-SP in these cells was inhibited by ZD6021 (0.1 nM–10 $\mu$M) in a concentration-dependent manner; $pD_{250} = 7.60 \pm 0.12$. At 100 nM antagonist, the inhibition of ASP-SP (100 nM)-evoked secretion was 76.0 $\pm$ 3.0% ($p < 0.001$).

Using preparations of isolated tissues, we then evaluated potency of ZD6021 for blockade of smooth muscle activation. The change in rabbit pulmonary artery contractile activity stimulated by the selective NK$_1$ or NK$_2$ receptor agonists ASP-SP or BANK, respectively, was blocked by ZD6021. Increasing concentrations of ZD6021 elicited parallel rightward shifts of the respective agonist concentration-response curves (Fig. 3). Schild analyses yielded a $pA_2$ value for ZD6021 of 8.68 (slope = 0.87 $\pm$ 0.19; $n > 7$, nonsignificant compared with unity) against ASP-SP-mediated relaxation and a $pA_2$ value of 8.52 (slope = 0.91 $\pm$ 0.16; $n > 6$, nonsignificant compared with unity) against BANK-induced contractions. For both receptors, the data indicated competitive antagonism by ZD6021.

Relaxation of isolated human pulmonary artery by SP was also antagonized by ZD6021. The calculated $pK_B$ averaged about 8.51 and was independent of drug concentration; $pK_B = 8.7 \pm 0.3 (n = 5)$ and $8.3 \pm 0.3 (n = 7)$ at 0.1 and 1.0 $\mu$M, respectively. Cumulative concentrations of NKA resulted in progressive contraction of the isolated human bronchus, which was antagonized by ZD6021. In this case, the calculated $pK_B$ values were 7.2 $\pm$ 0.2 ($n = 6$) and 7.4 $\pm$ 0.1 ($n = 8$) at 0.1 and 1 $\mu$M ZD6021.

Progressive contraction of the isolated guinea pig ileum stimulated by increasing concentrations of the NK$_2$ receptor agonist senktide was dose dependently affected by ZD6021 (Fig. 4). As the concentration of ZD6021 was increased (1 $\times$ 10$^{-8}$–1 $\times$ 10$^{-5}$ M), the concentration-response curves were displaced to the right. Substantial inhibition was found at 10 nM ZD6021. In the presence of ZD6021, however, the maximum senktide-induced contractile response was markedly suppressed and this effect was independent of antagonist concentration. These results indicated that, in this preparation, antagonism by ZD6021 was not purely competitive.

In Vivo Pharmacology. The blockade of NK$_1$ or NK$_2$ receptor activation by ZD6021 was evaluated by determining its ability to prevent tracheal extravasation of Evans blue dye (NK$_1$ only) and changes in airway mechanics (NK$_1$ and NK$_2$). For the former assay, a single agonist dose (ASM-SP = 0.1 nmol/kg i.v.) was given to each animal that had been treated either with vehicle or ZD6021. In animals given the vehicle, this dose resulted in near maximal levels of extravasation, i.e., ED$_{90}$. The agonist-mediated response was dose

![Fig. 2. Inhibition of ligand binding to cloned human tachykinin receptors by ZD6021. MEL cell membranes with human recombinant NK$_1$ (squares), NK$_3$ (circles), and NK$_2$ (triangles) receptors were incubated with ~1 nM of the indicated ligand in the presence of increasing [ZD6021]. Nonspecific binding was determined with 1 $\mu$M of the homolog ligand. Values, expressed as percentage of control in duplicate, represent means $\pm$ S.E.M. ($n = 3–10$).](image-url)

![Fig. 3. Dose-dependent antagonism of NK$_1$- and NK$_2$-mediated changes in contractile activity of rabbit pulmonary artery. Effect of ZD6021 on concentration-response effects to ASM-SP (panel A) or BANK (panel B) in isolated rabbit pulmonary artery. Contraction of the pulmonary artery was stimulated by BANK in tissues denuded of endothelium. Values are means $\pm$ S.E.M. and are expressed as a percentage of the maximum response to either 100 $\mu$M papaverine for ASM-SP-induced relaxation responses or 30 mM BaCl$_2$ for BANK-stimulated contraction. The slopes from Schild regression (insets) are not significantly different from unity.](image-url)
dependently inhibited by ZD6021 (Fig. 5A). Half-maximal protection (ED$_{50}$) was determined to be at doses of 0.005 μmol/kg i.v. and 0.83 μmol/kg p.o. The feeding state of the animals did not markedly influence the level of protection offered by ZD6021. After an 18-h fast, administration of ZD6021 (1 μmol/kg p.o.) resulted in a level of protection similar to that found in animals permitted food ad libitum; i.e., 62.8 ± 10.1% in fasted animals versus 74.9 ± 5.6% in fed ones (n = 7 for both groups).

Cumulative intravenous administration of the selective tachykinin receptor agonists ASM-SP and BANK has been shown to elicit progressive decreases of $G_L$ in guinea pigs (Buckner et al., 1993). In the present studies, this loss of airway conductance was antagonized by intravenous or oral treatment of guinea pigs with ZD6021. Although ZD6021 did not alter baseline conductance, it markedly increased the dose of ASM-SP or BANK required to produce a 50% fall in baseline conductance (agonist ED$_{50}$). In the absence of ZD6021, the ED$_{50}$ doses for the selective NK$_1$ and NK$_2$ receptor agonists were 0.025 ± 0.007 (n = 4) and 0.28 ± 0.003 (n = 13) nmol/kg i.v., respectively. In the presence of antagonist, there was a dose-dependent, rightward displacement of the dose-response curves (data not shown). Consequently, the agonist ED$_{50}$ values increased by severalfold: ED$_{50}$ = 4.85 ± 0.74 (n = 6) nmol/kg i.v. for the NK$_1$ receptor and 2.15 ± 0.33 (n = 6) nmol/kg i.v. for the NK$_2$ receptor in response to ZD6021 (10 μmol/kg i.v.). The dose ratio for the NK$_1$ and NK$_2$ receptors was 194 and 77 (agonist ED$_{50}$ plus ZD6021 agonist ED$_{50}$ minus ZD6021 or control animals). By raising the dose of ZD6021 from 0.1 to 1.0 and to 10 μmol/kg i.v., the dose ratio for the NK$_2$ receptor increased from 1.4 (n = 3) to 11.8 (n = 3) and to 77 (n = 3).

In the studies described above, intravenous injection of BANK (0.1 nmol/kg) to control animals (given vehicle) produced a 90% fall in airway conductance. Decline to this severe level of bronchoconstriction was dose dependently prevented by treatment with ZD6021 (Fig. 5B). The dose of ZD6021 that resulted in only a 50% decrease in conductance from administration of this dose of BANK was defined as the ED$_{50}$. The values were 0.3 μmol/kg i.v. (data not shown) and 20 μmol/kg p.o. (2-h pretreatment period). In animals that did not receive the tachykinin receptor agonist, there was a 10% loss in conductance over the time course of the experiment. As a result, in animals treated with ZD6021, even at high doses, i.e., 100 μmol/kg p.o., conductance was not maintained at 100% of the initial baseline value.

In separate experiments, ZD6021 (10 μmol/kg i.v., n = 6) did not modify the bronchoconstriction produced by administration of cumulative intravenous doses of histamine.

The time-response effects of ZD6021 blockade of changes in both plasma extravasation and NK$_2$ receptor-mediated bronchoconstriction in guinea pigs were determined at doses of 3 and 30 μmol/kg p.o., respectively (Fig. 6). The agonist doses were set at 0.1 nmol/kg i.v. For the NK$_1$ receptor, the pharmacodynamic half-life was 280 min (maximum protection = 150 min, $r^2 = 0.47$), whereas for the NK$_2$ receptor, the $t_{1/2}$ was 458 min (maximum protection at 60 min, $r^2 = 0.58$). In both cases, modest protection remained at 12-h postadministration.

Chronic administration of ZD6021 did not result in signifi-
significant loss of protection against ASM-SP-mediated plasma extravasation. Animals were administered ZD6021 (3 μmol/kg p.o. b.i.d. or vehicle) for 4 consecutive days. On the 5th day, the animals were treated once and then readministered 72 h later either ZD6021 (1 μmol/kg p.o.) or vehicle and challenged with ASM-SP (0.1 nmol/kg i.v.). A 25% decrease (nonsignificant, \( p < 0.053, n = 9 \)) was found in the level of protection afforded by ZD6021 after chronic administration. This value, 63 ± 9%, was within experimental error of those previously obtained at this dose in separate studies (Fig. 5).

Capsaicin is a strong irritant that at low doses excites sensory C-fibers in the lung and releases both SP and NKA (Saria et al., 1985). Intravenous administration of capsaicin caused robust, dose-dependent bronchoconstriction in guinea pigs (Fig. 7). Separately administered, the selective NK1 receptor antagonist SR 140333 (1 μmol/kg i.v.) and the selective NK2 receptor antagonist ZD7944 (0.3 μmol/kg i.v.) did not prevent this adverse change. These doses given separately were sufficient to produce rightward shifts of ASM-SP- or BANK-mediated dose-response effects on airway conductance (dose ratios = 231 and 58, respectively). The levels of rightward displacement were similar to those found with ZD6021 (10 μmol/kg i.v.) for both agonists (data not shown).

When administered in combination, however, SR 140333 and ZD7944 shifted the dose-response effects of capsaicin to the right by 29-fold (\( p < 0.05 \) versus vehicle \(-\log ED_{50}\)). In animals treated with only ZD6021 (10 μmol/kg i.v.), a similar shift (34-fold, \( p < 0.05 \)) of the capsaicin-response curve was obtained.

**Discussion**

The purpose of the present study was to characterize the pharmacological activity of a novel antagonist of all three tachykinin receptors. The major findings indicate that ZD6021 is a potent, orally active antagonist capable of attenuating tachykinin-mediated responses. The in vitro activity of ZD6021 was evaluated in three separate assay systems: ligand binding to the human cloned receptors, mucous secretion by cultured cells, and smooth muscle contractile function using isolated tissues. In all cases, the compound potently blocked agonist-mediated events in a dose-dependent manner. Potency values were highest from binding studies of the human cloned receptors. The \( K_i \) values for the human NK1 and NK2 receptors were both subnanomolar with about 5-fold greater affinity for the NK1 receptor. The affinity constant for the human NK2 receptor was about 2 orders of magnitude lower than that of the NK2 receptor. Nonetheless, at appropriate concentrations of ZD6021, inhibition of all three receptors can be gained, thereby providing a useful pharmacological tool for examination of disease processes such as asthma.

Although the strong antagonist-receptor interaction was reflected in favorable response inhibition, potency values of ZD6021 were markedly reduced in some cases compared with those obtained in binding assays. For example, the pIC\(_{50}\) obtained in the mucous secretion assay was only 7.6, whereas the pK\(_i\) was greater than 9.0 in binding studies for the NK1 receptor. The former value was similar to that found for the selective NK1 receptor antagonist SR 140333 in the same assay (pIC\(_{50} = 8.0\), Caccese et al., 1999) but this compound...
also showed subnanomolar affinity in binding assays (Emonds-Alt et al., 1993). Other workers have also reported discrepancies of potency values between different assays for the NK₁ receptor as well as differences in the manner of antagonism, i.e., competitive versus noncompetitive. Goldhill et al. (1999) reported competitive antagonism by SR 140333 of SP-induced secretion from colon epithelial tissue and non-competitive blockade of SP-mediated contraction of isolated ileum. Inhibition by SR 140333 of SP binding to rat brain NK₁ receptors was also found to be competitive but inhibition of agonist-mediated relaxation of isolated rabbit pulmonary artery was noncompetitive (Emonds-Alt et al., 1993). Construction of the assay conditions, e.g., the origin of the tissue, seemed to impact the outcome of the responses and the potency values.

Since the pharmacology of the human NK₁ and NK₂ receptors measured in vitro closely resembles the biological activity found in rabbit tissues (Coge and Regoli, 1994), we chose to profile the pharmacology of ZD6021 in tissues from these species. Potency of ZD6021 for the NK₁ receptor was similar between species, consistent with earlier work. For example, the dissociation constant of the competitive NK₁ receptor antagonist CP 99994 is in the nanomolar range in both isolated human pulmonary artery (Corboz et al., 1998; Pedersen et al., 2000) and rabbit vena cava (Coge and Regoli, 1994). Potency determinations of compounds such as SR 140333, which display noncompetitive antagonism, are also comparable in tissues prepared from the two species (Emonds-Alt et al., 1993; Pedersen et al., 2000).

Unlike the potency measures of ZD6021 for the NK₁ receptor, the NK₂ receptor values obtained from human and rabbit tissues markedly differed, by an order of magnitude. These results were surprising based on previous studies. Contrac-
tion of the rabbit pulmonary artery or isolated human bronchus was competitively blocked by the selective NK₂ receptor antagonist SR 48968, resulting in pKₐ₅₀ values of about 9 (Advenier et al., 1992). In our hands, the potency of SR 48968 was also similar; pKₐ₅₀ values = 9.3 and 9.0 for rabbit pulmo-

nary artery and human bronchus, respectively (data not shown). The isolated human pulmonary artery provides a system free of contaminating contractile effects mediated by NK₂ or NK₁ receptors (Pedersen et al., 2000), and tachyki-

nin-induced contraction of isolated human bronchus is medi-
ated only by NK₂ receptors (Sheldrick et al., 1995). There-

fore, the reason for the disparity between the sets of results with ZD6021 for the NK₂ receptor for the two species is not readily apparent. Of note, however, are major discrepancies, i.e., 2 to 3 orders of magnitude, that have been detected between binding affinities for the human neurokinin receptors and pharmacological responses in human tissues (Pedersen et al., 2000).

The change of vascular integrity following tissue exposure to substance P has long been accepted as a valid method for evaluating potency of tachykinin antagonists. Substance P gives rise to extravasation of plasma proteins in a number of tissues (Lembeck and Holzer, 1979), and this effect has been linked to allergic airway responses (Saria et al., 1983). Changes in vascular permeability occur by the formation of gaps between endothelial cells of postcapillary venules (Baluk, 1997). Deletion of the gene encoding neutral endopeptidase results in widespread basal plasma extravasation, which can be restored upon treatment with NK₁ receptor antagonists (Lu et al., 1997). Extravasation produced by pharmacological or physiological stimuli has been blocked by pretreatment with selective NK₁ receptor antagonists. Reported values (ED₅₀) were 10 μg/kg p.o. for CP 122,721 in guinea pig lung (McLean et al., 1996), 7 μg/kg i.v. for SR 140333 in rodent skin (Emonds-Alt et al., 1993), and 150 μg/kg i.v. for RP 67580 in a similar preparation (Inoue et al., 1996). Comparatively, ZD6021 provided a similar level of blockade (ED₅₀ = 500 μg/kg p.o.) or was more active (ED₅₀ = 3 μg/kg i.v.), dependent upon the route of administration, than these selective NK₁ receptor antagonists. Moreover, the activity of ZD6021 was well sustained after oral delivery; the pharmacodynamic t₁/₂ was 281 min with peak activity occurring at 150 min.

Administration of either substance P or neurokinin A by inhalation or intravenous injection produces bronchocon-
striction, which can be measured as labored breathing (dys-
pnea) or a decrease in pulmonary function in guinea pigs (Kusner et al., 1992; Buckner et al., 1993). Pretreatment with inhibitors of neutral endopeptidase exacerbates this response in guinea pigs (Kusner et al., 1992; Savoie et al., 1995) and humans (Cheung et al., 1993). In guinea pigs, blockade of the NK₂ receptor caused the dose-response curves elicited by NK₂ receptor agonists to shift to the right. This shift was more pronounced, by as much as one log unit, in the presence of a selective NK₁ receptor antagonist, suggesting that NK₂ receptor agonists (including BANK) at high doses could produce bronchoconstriction via NK₁ receptor activation (Buck-

ner et al., 1993). We did not pretreat the animals with addi-
tional selective NK₁ or NK₂ antagonists during the evaluation of ZD6021. The agonist-mediated bronchocon-
striction was markedly displaced to the right in the presence of ZD6021 (by 194- and 77-fold at 10 μmol/kg i.v.) and these changes are consistent with the in vitro potency profile of the compound.

Several studies have reported that additive or synergistic benefits were gained by blocking both the NK₁ and NK₂ receptors. During bronchoconstriction, evoked by antidromic vagal stimulation, either no protection or only partial blockade resulted from selective antagonism of the NK₁ or NK₂ receptor, respectively, whereas cessation of the airway response was brought about by combination treatment with CP 99,994 and SR 48,968 (Savoie et al., 1995). Similarly, a synergistic reduction of airway hyper-responsiveness and inflammatory cell infiltration in ascaris-sensitized monkeys has been reported using these antagonists in combination (Turner et al., 1996). Vagal nerve stimulation of plasma extravasation in the guinea pig lung was mostly prevented by pretreatment with CP 99,994, but leakage was lowest in the presence of both the NK₁ receptor antagonist and the NK₂ receptor antagonist SR 48,968 (Savoie et al., 1995). In the present investigation, low doses of either SR 140333 or ZD7944 (NK₂ receptor antagonist) did not prevent capsaicin-mediated bronchoconstriction. In combination, however, they resulted in a marked rightward displacement of the capsaicin-response curve and this change was similar to that obtained using ZD6021 alone. These data suggest that in the guinea pig and other species, preservation of airway function is best achieved by blockade of both NK₁ and NK₂ receptors during pathophysiologica l conditions.

The compound MDL 105,212A was the first reported nonpeptide orally available antagonist with high affinity for both NK₁ receptors.
and NK₂ receptors (Kudlacz et al., 1996). Affinity values (pA₂) for the NK₁ and NK₂ receptors were 8.2 and 8.7, respectively. Capsaicin-induced dyspnea in conscious guinea pigs was blocked after oral administration (ED₅₀ = 50 mg/kg). In our hands, MDL 105,212A yielded pKᵦ values for the NK₁ and NK₂ receptors of about 7.5 and 6.8 (rabbit pulmonary artery) and 6.9 and 6.4 (human tissue), respectively. In addition, a dose of 19 mg/kg p.o. (or 30 μmol/kg) did not significantly attenuate the fall in airway conductance elicited by administration of ASM-SP or BANK to guinea pigs. By comparison, ZD6021 was a more potent antagonist of these tachykinin-mediated actions in the guinea pig lung. Gerspacher et al. (2000) have recently reported a novel compound, N-[R(R,R)-E]-1-(4-chloro-benzyl)-3-(2-oxo-azepan-3-ylcarbamoyl)-allyl]-N-methyl-3,5-bis-trifluoro-methyl-benzamide, that potently blocked agonist-mediated bronchoconstriction with ED₅₀ values of 0.03 and 0.73 mg/kg p.o. for the NK₁ and NK₂ receptors, respectively.

The present study also showed that ZD6021 had good affinity for the human NK₂ receptor, albeit considerably less than that demonstrated for the NK₁ or NK₂ receptors. Nonetheless, this compound potently blocked contraction of the longitudinal muscle of the guinea pig ileum. The pattern of antagonism was unusual; a dose-dependent shift to the right was accompanied by a suppression of the magnitude of contraction even at nanomolar concentrations. These data indicated that ZD6021 demonstrated strong antagonism of the NK₂ receptor, but in a manner distinct from the competitive type observed for the other two tachykinin receptors.

The functional role of the NK₂ receptor in the airways is unclear, although some mention of its biological effects seems warranted, especially with reference to cough. Antitussive effects of NK₂ receptor antagonists have been reported in several studies (for review, see Advenier and Emonds-Alt, 1996). However, the selective NK₂ receptor antagonist SR 124821 was shown to inhibit cough induced by aerosolized citric acid in guinea pigs (Daou et al., 1998). The site of action for this compound was not likely a direct effect on bronchial smooth muscle (Ellis et al., 1993), rather it is more probable that blockade resulted via neural control of respiratory events. As demonstrated by Bolser et al. (1997), tachykinergic regulation of the cough reflex, stimulated by mechanical irritation of the trachea, occurs within the central nervous system. Although we did not test the effects of ZD6021 in a model of cough, future studies with this compound may provide further clarification of NK₂ receptor involvement in the cough reflex.

The lung is exposed to a number of environmental irritants. In response to these invading particles, unmyelinated C-fibers innervating the lower airways release the tachykinins SP and NKA from their terminal endings, promoting mucous secretion and bronchoconstriction to aid expulsion and to prevent further particle distribution. Moreover, NKB is associated with the cough reflex (Daou et al., 1998). Alterations of the tachykinergic system result from chronic irritation of the lung, e.g., inhalation of cigarette smoke or from inflammatory diseases, such as asthma. These changes include 1) up-regulation of both NK₁ and NK₂ receptor mRNA in human lungs (Adcock et al., 1993; Bai et al., 1995); 2) decreased levels of neutral endopeptidase, which regulates the availability of the tachykinins (Di Maria et al., 1998); and 3) increased bronchial responsiveness to administration of exogenous SP or NKA (Cheung et al., 1993, 1994). Accordingly, the tachykinins may produce deleterious effects in such pathophysiological conditions and the tachykinergic system may offer an appropriate therapeutic target (Barnes, 1992). Future studies of asthma, cough, or other respiratory (patho)physiology may benefit from tachykinin antagonists, either selective for each of the tachykinin receptors, or those with affinity for multiple tachykinin receptors such as ZD6021.

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