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# Correlation between binding characteristics and functional antagonism in human glioma cells by tachykinin $NK_1$ receptor antagonists

Carla Palma *, Federica Nardelli, Stefano Manzini

*Department of Pharmacology, Menarini Ricerche, Via Tito Speri, 10, 00040 Pomezia, Rome, Italy*

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**Abstract**

Binding characteristics and functional antagonism exerted by two structurally related tachykinin $NK_1$ receptor antagonists, MEN 11467 ((1*R*,2*S*)-2*N*[1(*H*)indol-3-yl-carbonyl]-1-*N*-{$N^{\alpha}$(*p*-tolylacetyl)-$N^{\alpha}$(methyl)-D-3-(2-naphthyl)alanyl}diaminocyclohexane) and FK888 ($N^2$-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-L-3-(2-naphthyl)alaninamide), were investigated in the human astrocytoma cell line U373 MG. In radioligand binding studies, conducted with [$^3$H]substance P and intact cells at 37°C, MEN 11467 bound to tachykinin $NK_1$ receptors in an irreversible manner with a $K_i$ value of $1.2 \pm 0.5$ nM while FK888 bound in competitive manner with a $K_i$ value of $4.6 \pm 2.2$ nM. Receptor blockade by both antagonists resulted in a powerful and complete inhibition of functional responses induced by substance P, such as accumulation of the second messenger inositol monophosphate or interleukin-6 release. However, MEN 11467 showed a greater potency for blocking functional responses than FK888 despite their similar affinity for human tachykinin $NK_1$ receptors. Moreover, MEN 11467 was more potent in inhibiting late rather than early phases of substance P-induced inositol monophosphate accumulation and its antagonism was enhanced by drug preincubation and barely affected by removal of unbound drug from the external medium, suggesting that MEN 11467 bound in a tight manner to the receptor. Such behaviour was not observed with the competitive and rapidly reversible antagonist FK888. These data indicate that the small differences in the chemical structure of MEN 11467 and FK888 determine the different binding characteristics at the tachykinin $NK_1$ receptor and which are responsible for the greater potency of MEN 11467 for blocking functional responses. The $K_i$ value obtained in binding studies may be inadequate to reveal the real potency of tachykinin $NK_1$ receptor antagonists. 

*Keywords:* [$^3$H]Substance P binding; Inositol phosphate; Tachykinin $NK_1$ receptor antagonist; Glioma cell, human

## 1. Introduction

The tachykinin $NK_1$ receptor mediates a wide range of physiological and potentially pathological responses to tachykinins (Quartara and Maggi, 1998). On the basis of this fact, a great deal of research has been carried out to identify and develop a specific, selective and long-lasting antagonist of this class of receptor as a potential new drug for the treatment of tachykinin-related pathologies. In the last decades a wide range of tachykinin $NK_1$ receptor antagonists have been described with heterogeneous chemical structures and types of pharmacological antagonism (Quartara and Maggi, 1998). To characterize their pharmacological profile, most tachykinin $NK_1$ receptor antagonists have been studied in similar experimental models ranging from binding studies in cells expressing human tachykinin $NK_1$ receptors (IM-9, U373, transfected Chinese hamster ovary (CHO)) to the assessment of in vitro or in vivo blockade of substance P-induced functional responses (Johnson and Johnson, 1992; Heuillet et al., 1993; Goso et al., 1994; Sagan et al., 1997). However, comparable studies to assess the affinity and the characteristics of interaction with the receptor in binding experiments and the blockade of functional responses induced by substance P stimulation in the same experimental model, and in particular with the human tachykinin $NK_1$ receptor type, are lacking. To investigate this issue we decided to study, in the human astrocytoma cell line U373 MG, the binding interaction and the antagonism of the functional cellular response to substance P of two tachykinin $NK_1$ receptor antagonists: the peptide FK888 ($N^2$-[(4*R*)-4-hydroxy-

* Corresponding author. Tel.: +0039-06-91184463; fax: +0039-06-9100220

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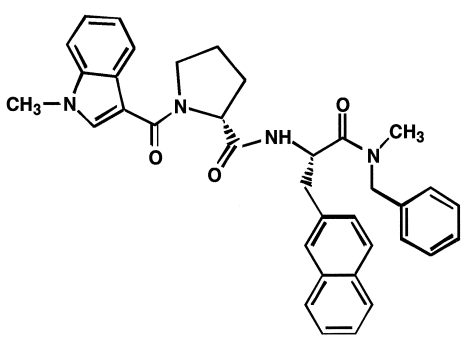
1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-L-3-(2-naphthyl)alaninamide) (Fujii et al., 1992) and the structurally related pseudopeptide, MEN 11467 ((1*R*,2*S*)-2 *N*[1(*H*)indol-3-yl-carbonyl]-1-*N*-{$N^{\alpha}$(*p*-tolylacetyl)-$N^{\alpha}$(methyl)-D-3-(2-naphthyl)alanyl}diaminocyclohexane) (Cirillo et al., 1998a). MEN 11467 is the result of the stepwise modification of the peptide backbone of the pseudo-peptide analogue MEN 10725 (Sisto et al., 1995), derived from FK888. MEN 11467 is characterized by a 1,2 (1*R*,2*S*) *cis*-diaminocyclohexane moiety and a D-(2-naphthyl)alanine residue inserted in the peptide chain in a reversed direction, providing two consecutive retro-inverso peptide amide bonds starting from the C-terminus. The introduction of these modifications maintains the three-dimensional assembly of the aromatic rings that is crucial for biological activity (Pispisa et al., 1996) (Fig. 1). U373 MG cells were selected for the following reasons: (a) they express a large amount of functionally coupled human tachykinin $NK_1$ receptors (Lee et al., 1989), but no other tachykinin receptor subtypes (Heuillet et al., 1993); (b) the biochemical pathways activated following receptor stimulation are well established, being related to the generation of intracellular second messengers such as inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (Lee et al., 1992), (c) complex final biological responses (release of soluble mediators such as taurine and cytokines) have been correlated to 1,2-diacylglycerol-induced protein kinase C activation (Lee et al., 1992; Palma et al., 1995; Palma and Manzini, 1998) and finally (d) tachykinin $NK_1$ receptors expressed on astrocytes may participate in pathological conditions such as multiple sclerosis and brain tumors (Kostyk et al., 1989; Palma et al., 1999). The effects of the tachykinin $NK_1$ receptor antagonists were evaluated on two related, but distinct functional responses triggered by substance P: the rapidly occurring formation of inositol monophosphate and the release of interleukin-6 as a complex event that requires activation of a cascade of intracellular signal(s) affecting gene expression, mRNA stability and protein synthesis (Palma et al., 1995; Palma and Manzini, 1998). In addition, the nature of binding antagonism of the compounds was determined in competition and saturation studies.

To allow a real correlation of binding and functional responses both types of experiments were performed at the same physiological temperature (37°C) in metabolically active cells to ensure the appropriate dynamic interaction between ligands, receptor, G-proteins and second messengers. Preliminary experiments indicated that suitable equilibrium binding conditions are achieved.

Evidence has been gained that binding characteristics influence the intensity and duration of the antagonist blockade of functional responses in a manner that could affect the therapeutic actions of these drugs.

FK888



MEN 11467

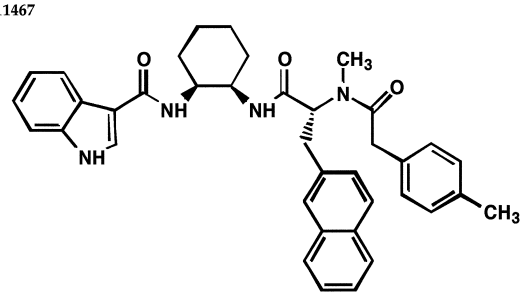


Fig. 1. Chemical structures of MEN 11467 and FK888.

## 2. Materials and methods

### 2.1. Drugs

The compound FK888 ($N^2$-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-L-3-(2-naphthyl)alaninamide) was purchased from Tocris Cookson, Bristol, England; MEN 11467 ((1*R*,2*S*)-2 *N*[1(*H*)indol-3-yl-carbonyl]-1-*N*-{$N^{\alpha}$(*p*-tolylacetyl)-$N^{\alpha}$(methyl)-D-3-(2-naphthyl)alanyl}diaminocyclohexane) was synthesized at the Chemistry Department of Menarini Ricerche, Pomezia, Italy (Fig. 1). Both drugs were dissolved at a concentration of $10^{-2}$ M in dimethyl sulfoxide and stored at −20°C. Dilutions were then made in the appropriate experimental buffers.

### 2.2. Culture of human astrocytoma U373 MG cells

The human astrocytoma cell line U373 MG was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated (65°C, 30 min) foetal bovine serum with 5 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (foetal bovine serum medium) at 37°C in an atmosphere of 5% $CO_2$.

### 2.3. Substance P receptor binding assays with U373 MG intact cells at 37°C

Binding assays with confluent adherent intact cells were conducted in 24-well plastic dishes, as described previ-

ously (Goso et al., 1994) with some modifications. Briefly, U373 MG cells were seeded in 24-well tissue culture dishes at $2.5 \times 10^5$ cells/well and allowed to adhere for 24 h at 37°C. Then the culture medium was removed and 500 μl of RPMI 1640 supplemented with 0.2% glucose and 1% bovine serum albumin was added to each well for 30 min. The buffer was then aspirated and fresh buffer containing [$^3$H]substance P (specific activity = 40 Ci/mmol; Amersham International, Little Chalfont, Buckinghamshire, UK) was added in a final volume of 500 μl. The non-specific binding was defined with unlabelled substance P at 10 μM. The plates were incubated at 37°C for the appropriate incubation period. The reaction was stopped by aspirating the medium and then rinsing each well three times with 1 ml of 0.9% NaCl in 10 mM HEPES (pH 7.2) wash buffer. The cells were solubilized in 5% sodium dodecyl sulphate in 0.01 M HCl (0.5 ml) at 37°C for 15 min and counted for radioactivity in a liquid scintillation counter.

In association studies, 0.3 nM [$^3$H]substance P was added to cells and the bound radioactivity was measured at various times ranging from 1 min to 3 h. In dissociation studies cells were incubated with 0.3 nM [$^3$H]substance P up to equilibrium (30 min), then the dissociation was initiated by the addition of an excess of unlabelled substance P (10 μM) and the bound radioactivity was measured at various times ranging from 1 min to 2 h.

In competition studies, U373 MG cells were incubated with 0.3 nM [$^3$H]substance P and various concentrations of tachykinin $NK_1$ receptor antagonists ($10^{-12}$–$10^{-5}$ M) at 37°C for 1 h.

To evaluate the nature of tachykinin $NK_1$ receptor antagonism in binding assays, series of saturation curves were recorded with [$^3$H]substance P (0.1–10 nM) in the absence or in the presence of MEN 11467 (1–10 nM) or FK888 (10 nM). Substance P binding assays were performed with U373 MG cells at 37°C for 1 h as described above.

### 2.4. Inositol phosphate accumulation

Assay conditions were based on the procedure of Berridge et al. (1982). In brief, U373 MG cells were seeded in 24-well tissue culture dishes at $2.5 \times 10^5$ cells/well and allowed to adhere for 24 h at 37°C. Then the culture medium was removed and the cells were labelled for 24 h with 1 μCi/well *myo*-[$^3$H]inositol (47 Ci/mmol, Du Pont de Nemours, NEN Products Division —Europe, Belgium). The cells were rinsed and incubated in 1 ml of bovine serum albumin buffer (PBS 1× w/o $Ca^{++}Mg^{++}$, Na-Hepes 20 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1.2 mM, EGTA 1 mM, D-glucose 11.1 mM, bovine serum albumin 0.05%) containing 10 mM of LiCl in the absence or the presence of substance P and tachykinin $NK_1$ receptor antagonists. Reactions were performed for the appropriate incubation times at 37°C in a humidified atmosphere and were terminated by aspiration of the buffer and the addition of 1 ml of ice-cold methanol/0.1 N HCl (2:1 vol/vol). The aqueous phase was removed and applied to an anion exchange column (AG 1-X8, Bio-Rad), and inositol monophosphate was eluted with 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was quantified by liquid scintillation spectrometry. In time course experiments cells were stimulated with substance P (100 nM) and reactions were stopped at various intervals ranging from 5 min to 24 h; in dose–response studies cells were stimulated with substance P (0.1–1000 nM) for 1 or 6 h; in inhibition studies the tachykinin $NK_1$ receptor antagonists were co-administered with substance P 100 nM for 1 or 6 h of incubation; in preincubation experiments tachykinin $NK_1$ receptor antagonists were added 1 h before substance P stimulation for 1 h; and in washing experiments cells were preincubated for 1 h with tachykinin $NK_1$ receptor antagonists and then were washed twice with 2 ml of bovine serum albumin buffer before substance P stimulation for 1 h, 6 h or 24 h.

### 2.5. Detection of interleukin-6 in supernatants of U373 MG cells

U373 MG cells were seeded in 24-well tissue culture dishes at $3.5 \times 10^5$ cells/well and allowed to adhere for 24 h at 37°C. Then the culture medium was removed, the cells were washed three times in serum-free RPMI and fresh medium containing foetal bovine serum was added. Cells were cultured for 18 h in the presence or absence of substance P (100 nM). The tachykinin $NK_1$ receptor antagonists were co-administered with the agonist. After the incubation time, the supernatants were collected and spun free of cells and debris. Interleukin-6 levels were assayed with a specific ELISA kit (Quantikine™, Research and Diagnostic System, Minneapolis, MN). The data were analysed by linear regression using the Microplate manager program (Bio-Rad Lab., Richmond, CA). In time

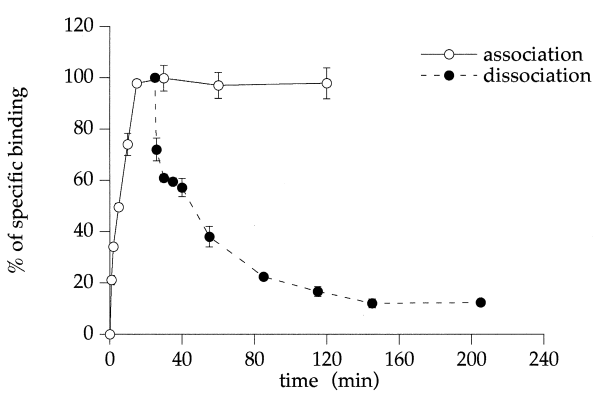



Fig. 2. Association and dissociation binding kinetics of [$^3$H]substance P. Association and dissociation binding kinetics of [$^3$H]substance P (0.3 nM) to human tachykinin $NK_1$ receptors were measured in intact adherent cells at 37°C. At the steady state for association kinetics (30 min), 10 μM unlabelled substance P was added to initiate dissociation. Data are presented as percent of specific binding and represent the means ± S.E.M. of triplicate determinations of one representative experiment out of three.

course experiments cells were stimulated with substance P at 100 nM and reactions were stopped at intervals ranging from 1 h to 48 h; in dose–response studies cells were stimulated with substance P (0.1–1000 nM) for 18 h.

### 2.6. Data analysis

Equilibrium binding parameters were computed by analysis of the binding isotherms according to the mass action law using non-linear least-squares fitting procedures with the computer program LIGAND (Munson and Rodbard, 1980). Binding data were fitted to a one-site model or to a two-site model to estimate binding affinity and capacity. Association/dissociation studies of substance P binding to tachykinin $NK_1$ receptors were analysed with the computer program KINETIC (Biosoft, Cambridge, UK).

Curves of inositol monophosphate formation and interleukin-6 release were fitted using the computer program ALLFIT (De Lean et al., 1978), which provides $IC_{50}$ values and automated statistical analysis.

## 3. Results

### 3.1. Kinetic studies of [$^3$H]substance P binding to tachykinin $NK_1$ receptors expressed on U373 MG adherent cells

Kinetic studies of [$^3$H]substance P binding to human tachykinin $NK_1$ receptors were performed with intact adherent U373 MG cells at 37°C. In association/dissociation kinetic analysis, after the appropriate incubation time (30 min), in which steady-state binding was achieved (Fig. 2), 10 μM unlabelled substance P was added to the medium to investigate the dissociation kinetics. The dissociation analysis of [$^3$H]substance P was statistically significant for two sites with the following dissociation constant $K^1_{-1} = 0.031 \pm 0.009\ \text{min}^{-1}$ and $t^1_{1/2} = 23.1$ min. From the association curve ($K_{obs} = 0.217 \pm 0.02\ \text{min}^{-1}$), $K^1_{+1} = 0.62 \times 10^9 \pm 0.05 \times 10^9\ \text{M}^{-1}\ \text{min}^{-1}$ was determined (Fig. 2). From these data, a $K_{d1} = 0.05 \pm 0.01$ nM was calculated for the first site, which accounted for 85% of the receptor population. However, the second site, representing 15% of the whole receptor population, was very poorly defined. Therefore we report only the data for one site.

Table 1
Effects of tachykinin $NK_1$ receptor antagonists on saturation binding of [$^3$H]substance P to U373 MG $NK_1$ receptors

| Antagonist | $K_d$ (nM) | $B_{max}$ (sites/cell) |
|---|---|---|
| None | 0.67 ± 0.1 | 78 000 ± 5250 |
| MEN 11467 1 nM | 0.89 ± 0.1 | 65 850 ± 5625 |
| MEN 11467 10 nM | 1.99 ± 0.3[a] | 47 475 ± 6975[b] |
| FK888 10 nM | 2.87 ± 1.1[a] | 70 875 ± 2182 |

Specific binding of [$^3$H]substance P in the absence and in the presence of tachykinin $NK_1$ receptor antagonists was measured in intact U373 MG cells at 37°C. Affinity constant ($K_d$) and binding capacity ($B_{max}$) for substance P were obtained through Scatchard analysis of the data. Values and statistics were calculated by using the computer program LIGAND.
[a] $P < 0.001$ as compared to control.
[b] $P < 0.05$ as compared to control.

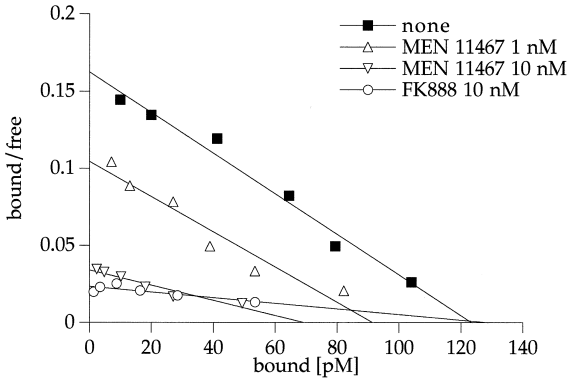



Fig. 3. Scatchard analysis of saturation isotherms of the binding of [$^3$H]substance P to tachykinin $NK_1$ receptors in U373 MG cells. U373 MG cells were incubated at 37°C for 1 h with increasing concentrations of [$^3$H]substance P in the absence or presence of the indicated tachykinin $NK_1$ receptor antagonists. Data shown are from one of three representative experiments run in triplicate.

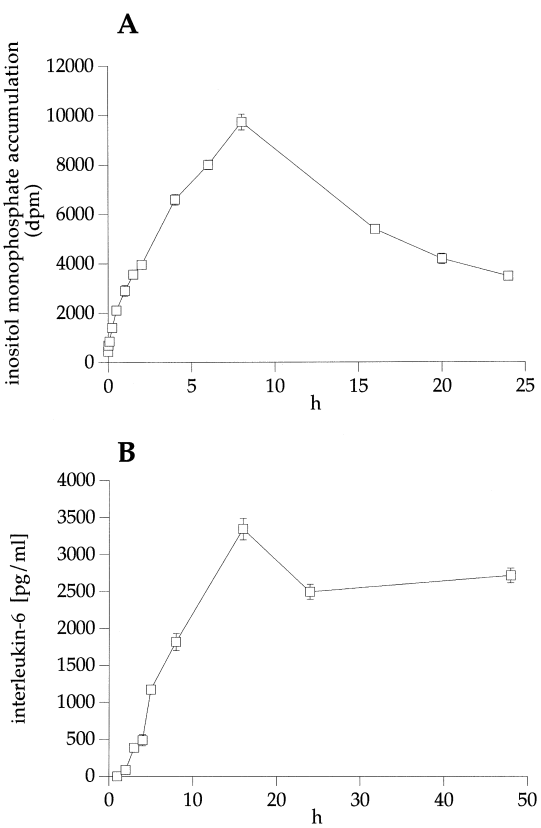



Fig. 4. Time-dependent inositol monophosphate accumulation and interleukin-6 secretion induced by substance P (100 nM) in U373 MG cells. U373 MG cells ($3 \times 10^5$ cells/well) were stimulated with 100 nM of substance P. Inositol monophosphate accumulation (A) and interleukin-6 release (B) were measured at the times indicated on the abscissa as described in Section 2. The data represent the means ± S.E.M. of three independent experiments for each response assayed.

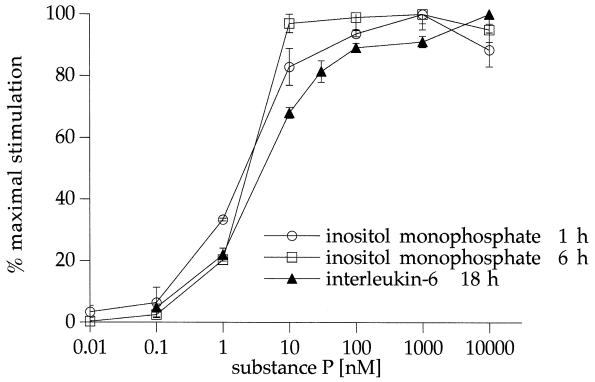



Fig. 5. Concentration dependence of substance P-induced inositol monophosphate accumulation or interleukin-6 release in U373 MG cells. U373 MG cells were stimulated with various substance P concentrations. Inositol monophosphate accumulation was measured after 1 h or 6 h of incubation, and interleukin-6 release after 18 h. Data are presented as percent of maximal stimulation of each functional response assayed (inositol monophosphate 1 h 8059 ± 276 dpm; inositol monophosphate 6 h 18008 ± 1216 dpm; interleukin-6 11795 ± 379 pg/ml) and represent the means ± S.E.M. of triplicate determinations of one representative experiment out of three.

The stability of steady-state binding up to 2 h and the rapid [$^3$H]substance P dissociation suggested that the incubation time of 1 h at 37°C was suitable to achieve adequate equilibrium binding conditions in cells maintained in a physiological state.

### *3.2. Competition studies of tachykinin NK$_1$ receptor antagonists on [$^3$H]substance P binding*

The affinities of MEN 11467 and FK888 for the human tachykinin NK$_1$ receptor were assessed by measuring the displacement of [$^3$H]substance P (0.3 nM) binding to U373 MG cells at 37°C for 1 h. Simultaneous analysis, by the computer program LIGAND, of binding isotherms obtained from three independent experiments indicated a high comparable potency for both MEN 11467 and FK888. The $K_i$ values obtained were 1.2 ± 0.5 nM and 4.6 ± 2.2 nM for MEN 11467 and FK888, respectively.

### *3.3. Analysis of the nature of antagonism in saturation studies of [$^3$H]substance P*

Scatchard analysis of the data for saturation binding of [$^3$H]substance P (0.1–10 nM) to tachykinin NK$_1$ receptors expressed in U373 MG cells at 37°C indicated a single population of binding sites for substance P with a $K_d$ value of 0.67 ± 0.1 nM and a $B_{max}$ of 78000 ± 5250 sites/cell ($n = 3$). The presence of MEN 11467 (10 nM) significantly decreased both substance P binding capacity and the dissociation constant, suggesting an irreversible interaction (Table 1 and Fig. 3). In contrast, FK888 (10 nM)-induced inhibition was competitive because the $K_d$ of the ligand was modified in the presence of the compound without significant modification of the number of receptor binding sites (Table 1 and Fig. 3).

### *3.4. Induction of inositol monophosphate accumulation and interleukin-6 release by substance P stimulation in U373 MG cells*

Stimulation of tachykinin NK$_1$ receptors expressed on U373 MG cells by substance P triggers inositol monophosphate accumulation and enhancement of basal interleukin-6 release (Palma et al., 1995). As shown in Fig. 4A, the accumulation of the second messenger inositol monophosphate induced by substance P 100 nM was very rapid. The response started at 5 min and peaked at 6–8 h. A decrease in the amount of inositol monophosphate accumulation was found at 18–24 h. By using a specific ELISA kit, a discrete amount of interleukin-6 (640 ± 90 pg/ml) was detected in the supernatants of unstimulated U373 MG cells. An increased interleukin-6 level was found in the culture supernatants as early as 3 h after substance P

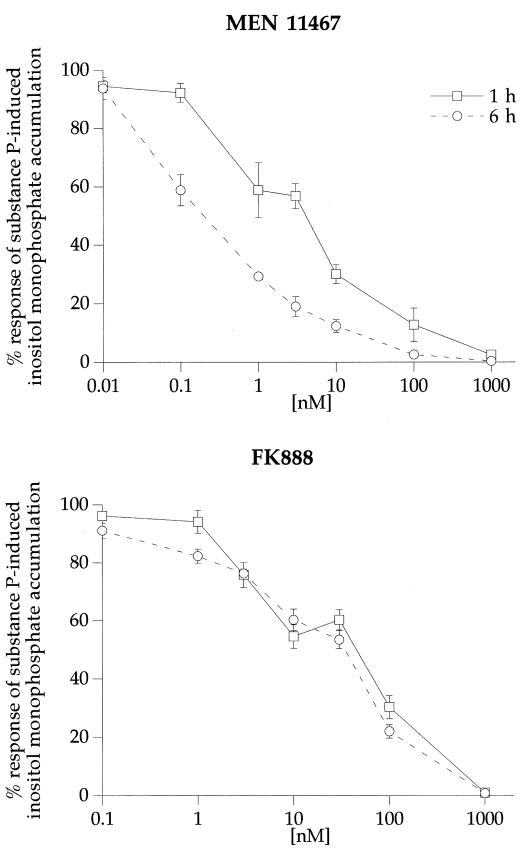



Fig. 6. Inhibition by tachykinin NK$_1$ antagonists of inositol monophosphate accumulation induced by substance P following 1 and 6 h of exposure. U373 MG cells were stimulated with substance P 100 nM in the presence or absence of tachykinin NK$_1$ receptor antagonists at the indicated concentrations. Inositol monophosphate accumulation was measured at 1 or 6 h following substance P addition. Data are presented as percent of response obtained in cells stimulated with substance P in the absence of the tachykinin NK$_1$ receptor antagonists. Data represent the means ± S.E.M. of three independent experiments.

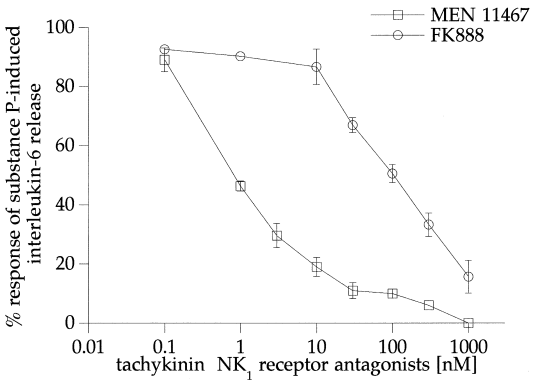



Fig. 7. Inhibition by tachykinin $NK_1$ receptor antagonists of substance P-induced interleukin-6 release. U373 MG cells were stimulated with substance P 100 nM in the presence or absence of various concentrations of the tachykinin $NK_1$ receptor antagonists.The presence of interleukin-6 in the culture supernatants was measured after 18 h of substance P stimulation. Data are presented as percent of the response obtained in cells stimulated with substance P in the absence of tachykinin $NK_1$ antagonists. Data represent the means ± S.E.M. of three independent experiments.

stimulation and the response peaked at 16 h. Despite a small decrease in interleukin-6 levels after 24 h, the interleukin-6 level remained stable until 48 h (Fig. 4B).

Both functional responses, measured at the peak (6 h for inositol monophosphate and 18 h for interleukin-6), were stimulated by substance P (0.1–1000 nM) in a similar concentration-dependent manner. The substance P concentration producing the half-maximal stimulation ($EC_{50}$) was $1.3 \pm 0.5$ nM and $3.8 \pm 0.5$ nM, for inositol monophosphate and interleukin-6, respectively (Fig. 5). The concentration–response curve for inositol monophosphate accumulation measured early after substance P stimulation (1 h) was parallel to that obtained at 6 h with an $EC_{50}$ value of $1.7 \pm 1$ nM (Fig. 5). Maximal activation of both functional responses occurred at 100 nM substance P and this concentration was selected to evaluate the potential inhibition by antagonists.

### *3.5. Tachykinin $NK_1$ receptor antagonist inhibition of substance P-induced inositol monophosphate accumulation: time dependence*

To investigate the potency of tachykinin $NK_1$ receptor antagonists in inhibiting functional responses triggered by substance P in U373 MG cells, inositol monophosphate accumulation was measured in an early phase (1 h) and at the peak (6 h) of substance P stimulation. U373 MG cells were treated with substance P 100 nM and different concentrations of tachykinin $NK_1$ receptor antagonists ($10^{-11}$–$10^{-5}$ M) were co-administered with the agonist. MEN 11467 as well as FK888 completely reversed substance P-induced inositol monophosphate accumulation and 'per se' had no effects on basal levels (data not shown), suggesting they can act as full antagonists. As shown in Fig. 6, MEN 11467 was more potent than FK888 in antagonizing tachykinin $NK_1$ receptor-triggered inositol monophosphate accumulation at both times. Simultaneous analysis of curves obtained in 3 independent experiments by using the computer program, ALLFIT, indicated that FK888 antagonized inositol monophosphate responses measured at 1 and 6 h of substance P incubation with equal $IC_{50}$ values of $27 \pm 9$ nM and $30 \pm 10$ nM, respectively. In contrast, MEN 11467 discriminated between early and late phases of substance P stimulation, inhibiting to a greater and significant extent ($P < 0.001$; one-way analysis of variance, ANOVA; Tukey–Kramer multiple comparisons test) the accumulation of inositol monophosphate after the longer (6 h) exposure to substance P ($IC_{50} = 0.2 \pm 0.08$ nM) that after the shorter (1 h) exposure to substance P ($IC_{50} = 2 \pm 0.8$ nM).

### *3.6. Effect of tachykinin $NK_1$ receptor antagonists on substance P-induced interleukin-6 release*

MEN 11467 and FK888 completely blocked the interleukin-6 secretion triggered by substance P (100 nM) for 18 h in U373 MG cells (Fig. 7), while they did not affect either basal or lipopolysaccharide-triggered interleukin-6 secretion, even at the highest concentration assayed ($10^{-5}$ M) (data not shown). Both tachykinin $NK_1$ receptor antagonists inhibited the substance P-induced response in a concentration-dependent manner with $IC_{50}$ values of $0.7 \pm 0.1$ nM and $47 \pm 7$ nM, for MEN 11467 and FK888, respectively (3 independent experiments) (Fig. 7). The inhibition curves and $IC_{50}$ values obtained with both antagonists were similar for inositol monophosphate accumu-

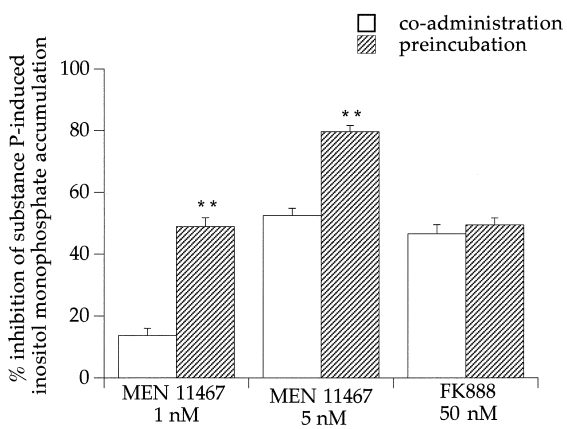



Fig. 8. Effect of tachykinin $NK_1$ receptor antagonist preincubation on substance P-induced inositol monophosphate accumulation in U373 MG cells. U373 MG cells were pretreated (dotted bars) or not (open bars) with tachykinin $NK_1$ receptor antagonist for 1 h before substance P 100 nM was added. Inositol monophosphate accumulation was measured after 1 h of substance P stimulation and data are presented as percent inhibition of the response obtained in cells stimulated with substance P in the absence of tachykinin $NK_1$ receptor antagonists. Data represent the means ± S.E.M. of triplicate determinations of one representative experiment out of two. $^{**}P < 0.001$ as compared to inhibition obtained with the corresponding tachykinin $NK_1$ receptor antagonist co-administration (one-way analysis of variance, ANOVA; Tukey–Kramer multiple comparisons test).

lation and interleukin-6 release measured at the peak of the substance P response.

### 3.7. Effect of tachykinin $NK_1$ receptor antagonist preincubation or removal from external medium on the blockade of substance P-induced inositol monophosphate accumulation

To further assess the interaction of MEN 11467 and FK888 with the human tachykinin $NK_1$ receptor, we investigated whether a 1-h preincubation could differentially affect the ability of these antagonists to inhibit substance P-induced inositol monophosphate accumulation. As shown in Fig. 8, drug preincubation significantly increased the antagonistic effect exerted by MEN 11467 (1 or 10 nM) while did not affect that produced by the competitive antagonist FK888 (50 nM).

In another set of experiments the 1-h antagonist preincubation was followed by extensive washing of the cells in order to remove the drugs from the external medium. Washed cells were then exposed to substance P and the ability to cause inositol monophosphate accumulation was assessed. As shown in Fig. 9, despite removal of unbound drug from the medium, the antagonistic effect of MEN 11467 was still largely unaffected at 1, 6 or even 24 h following substance P exposure. FK888 removal from the external medium caused a rapid and almost complete loss of activity (Fig. 9).

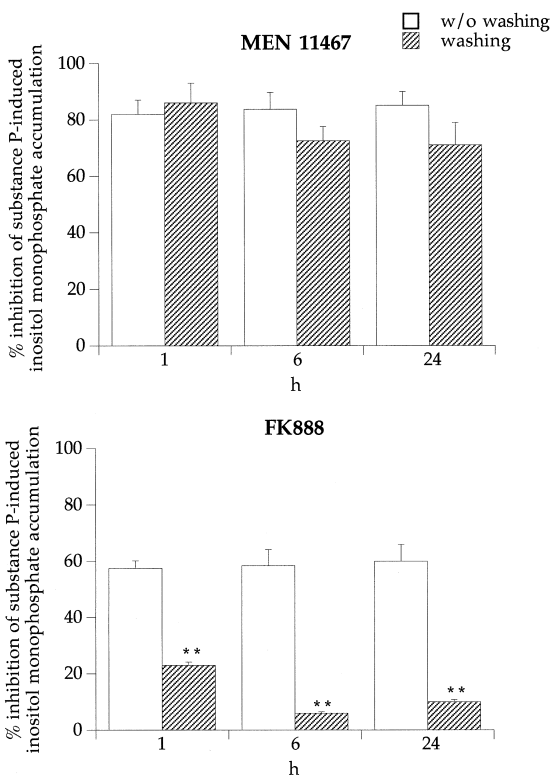



Fig. 9. Effect of removing unbound tachykinin $NK_1$ receptor antagonists on subsequent inhibition of substance P-induced inositol monophosphate accumulation. U373 MG cells were pretreated with tachykinin $NK_1$ receptor antagonist (MEN 11467, 5 nM; FK888, 50 nM) for 1 h. Cells were then thoroughly washed (dashed bars) or not (open bars) before substance P 100 nM was added. Inositol monophosphate accumulation was measured at 1 h, 6 h or 24 h of substance P stimulation and data are presented as percent inhibition of the response obtained in cells stimulated with substance P in the absence of tachykinin $NK_1$ receptor antagonists. Data are presented as the means ± S.E.M. of three independent experiments. $^{**}P < 0.001$ as compared to inhibition obtained with the corresponding tachykinin $NK_1$ receptor antagonists in unwashed cells (one-way analysis of variance, ANOVA; Tukey–Kramer multiple comparisons test).

## 4. Discussion

The astrocytoma cells U373 MG are an excellent tool to investigate the interaction and functional effects of substance P at the human tachykinin $NK_1$ receptor and to characterize the properties of specific receptor antagonists. To better compare the receptor occupancy with ligand-triggered signal transduction, we established the equilibrium conditions for radioligand binding studies with intact adherent U373 MG cells under the conditions used in the functional experiments. In the kinetic binding studies with intact U373 MG cells at 37°C, a very fast association of [$^3$H]substance P was observed. The dissociation was a more complex event since dissociation curves were best fitted with a biexponential decay model. However, the majority of tachykinin $NK_1$ receptors (85%) were in the more rapidly dissociating state. The different percentage of sites in the rapidly or the slowly dissociating state determined with the biexponential decay model of substance P was consistent with the different dissociation rates of substance P observed in various experimental models. In fact, Hanzhong et al. (1996) observed that only about 10% of radiolabelled substance P had dissociated from the receptor after 2 h in intact human tachykinin $NK_1$ receptor-transfected CHO at 4°C. Cascieri et al. (1992) described that the number of sites in the high-affinity slowly dissociating state varied from 50 to 90% among different human tachykinin $NK_1$ receptor-transfected CHO cell membrane preparations, and that the addition of the non-hydrolyzable GTP analog, guganylyl5′-(β,γ-imido)diphosphate, markedly altered the dissociation of substance P from the tachykinin $NK_1$ receptor by increasing the number of sites in the low-affinity, rapidly dissociating state. The experimental binding conditions used in this work can be strictly correlated with the functional activation of tachykinin $NK_1$ receptors since they were established for metabolically active cells. The incubation time of 1 h at 37°C was suitable to achieve adequate equilibrium binding conditions, as suggested by the stability of steady-state binding up to 2 h and the rapid dissociation of [$^3$H]substance P. In addition, the prompt substance P-induced internalization accompanied by rapid, long-lasting desensitization to substance P observed in rat endothelial cells (Bowden et al., 1994) probably does not influence [$^3$H]substance P binding under our experimental conditions. In fact, the $B_{max}$ capacity for [$^3$H]substance P in intact U373

MG cells measured at 37°C did not decrease compared to the $B_{max}$ value observed at 4°C, (Palma et al., 1999), indicating that the number of sites for substance P remained constant under conditions in which receptor internalization was or was not allowed to occur. Moreover, the substance P-triggered inositol monophosphate accumulation, an event strictly related to ligand-receptor occupancy, linearly increased up to 6–8 h in U373 MG cells in the continuous presence of substance P (immunoreactivity for substance P was present in a high amount in the supernatants of U373 cultured for at least 24 h, unpublished observation).

In competition binding studies with [$^3$H]substance P, FK888 and the close structurally related analogue, MEN 11467, bound with high affinity to tachykinin $NK_1$ receptors, showing a comparable potency ($K_i = 4.6 \pm 2.2$ nM and $K_i = 1.2 \pm 0.5$, respectively). The results obtained with MEN 11467 and FK888 in displacement experiments with human astrocytoma cells at 37°C were very similar to those obtained with tachykinin $NK_1$ receptors expressed on IM-9 cells of the human lymphoblastoma cell line at 22°C (Goso et al., 1994; Cirillo et al., 1998a). However, the characteristics of the interactions with the receptor were different for the two tachykinin $NK_1$ receptor antagonists. In [$^3$H]substance P saturation binding experiments, the presence of MEN 11467 significantly decreased substance P binding capacity and the dissociation constant, suggesting that this antagonist interacts with the receptor in an irreversible manner, likely due to its pseudo-irreversible or slow dissociation from tachykinin $NK_1$ receptors. As explained below, the results of functional studies of MEN 11467 antagonism are in agreement with this hypothesis. FK888 decreased only substance P affinity, showing a competitive and reversible mode of interaction with the tachykinin $NK_1$ receptor as already suggested in some functional studies (Fujii et al., 1992).

In contrast to IM-9 cells, in which the number of tachykinin $NK_1$ receptor sites is too low to give measurable functional responses, U373 MG cells respond to substance P stimulation by activating various pathways (Lee et al., 1992; Luo et al., 1996; Palma and Manzini, 1998). The turnover of phosphatidyl inositol is the initial and main event following substance P interaction with tachykinin $NK_1$ receptors (Lee et al., 1992). U373 MG cells showed a long-lasting response to substance P stimulation and inositol monophosphate accumulation increased for 6–8 h. This prolonged response to agonist give us the opportunity to evaluate the tachykinin $NK_1$ receptor antagonist on early and late phases of substance P stimulation.

Receptor blockade by both antagonists resulted in a powerful and complete inhibition of substance P-triggered responses such as the formation of inositol monophosphate and the multi-step event of interleukin-6 secretion. These inhibitions were specific and selective since tachykinin $NK_1$ receptor antagonists did not alter either the basal level of inositol monophosphate or interleukin-6 release nor lipopolysaccharide-induced interleukin-6 secretion, suggesting also that they acted as full antagonists. However, despite their similar affinity in binding studies, MEN 11467 showed a greater ability to block functional responses than FK888. In the experiments of inhibition of substance P-induced inositol monophosphate or interleukin-6 accumulation, when agonist and antagonists were co-administered, the $IC_{50}$ values obtained with MEN 11467 were one or two orders of magnitude lower than those obtained with FK888. The small differences between the chemical structures of MEN 11467 and FK888 could render MEN 11467 able to dissociate from the receptor in a more slowly reversible manner. Functional antagonism experiments confirmed the tight nature of MEN 11467 binding to the human tachykinin $NK_1$ receptor, as already highlighted in saturation binding experiments. In fact, MEN 11467 antagonism of substance P-triggered inositol monophosphate accumulation was enhanced by drug preincubation and was barely affected by removal of unbound drug from the external medium. The blockade of tachykinin $NK_1$ receptors by MEN 11467 lasted for more than 24 h in the absence of drug in the external medium. MEN 11467 antagonism does not require the continuous presence of MEN 11467 in the medium to compete with substance P for tachykinin $NK_1$ receptors. The different interactions of MEN 11467 and FK888 with the tachykinin $NK_1$ receptor are responsible for their antagonist potency. The competitive and rapidly reversible antagonist FK888 easily dissociated from the receptor following washing, losing the ability to prevent activation of the receptor by substance P. Moreover, the preincubation of U373 MG cells with FK888 did not ameliorate its antagonist potency. In addition, MEN 11467 was more potent in inhibiting the late rather than the early phase of substance P-induced inositol monophosphate accumulation whereas FK888 was equipotent in the two phases.

Thus, it is conceivable that for a competitive antagonist, which is characterized by a rapid dissociation from the receptor and continuous competition with the agonist for receptor occupancy, the concentration in the external medium is the major determinant of its degree of antagonism. Irreversible antagonism is characterized by a progressive and time-dependent increase in receptor blockade and, later, the effect of the antagonist will become largely independent of a decrease in drug concentration in the external medium. The correlation between binding characteristics and functional antagonism observed for FK888 and MEN 11467 was noted also for other tachykinin $NK_1$ receptor antagonists. The competitive compound MEN 10930 (Astolfi et al., 1997) showed a low degree of antagonism in blocking substance P-induced inositol monophosphate or interleukin-6 accumulation in U373 MG cells relative to its affinity for human receptor. Moreover, it does not discriminate between early and late phases of substance P-induced inositol monophosphate accumulation and preincubation did not increase its potency (unpub-

lished observation). In contrast, irreversible or non-competitive tachykinin $NK_1$ receptor antagonists, such as MEN 11149 and CP 122,721 (McLean et al., 1996; Cirillo et al., 1998b), showed a behaviour similar to that of MEN 11467 with a high antagonist potency, ability to discriminate between early and late phases of substance P stimulation, prolonged blockade of receptors after washing, and increase in antagonist efficacy following drug preincubation (unpublished observation). It has been already suggested that the non-competitive antagonism of CP 122,721 is responsible for its potent and long-lasting blockade of substance P-induced plasma extravasation in guinea pig lung and ureter (McLean et al., 1996).

In conclusion, irreversible/non-competitive interactions with the human tachykinin $NK_1$ receptor correlate with powerful and long-lasting inhibition of functional responses elicited by substance P. This type of drug-receptor interaction could lead to a prolonged in vivo pharmacological receptor blockade in spite of a decrease in plasma drug levels. A similar behaviour has been already observed with another irreversible tachykinin receptor antagonist, SR 48968, for inhibition of neurokinin A-induced bronchoconstriction in asthmatic patients (Van Schoor et al., 1998).

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