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Antagonistic properties of [Phe^3,Leu^{13}]porcine motilin

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Abstract

We describe the antagonistic properties due to the replacement of Pro^3 by phenylalanine in porcine motilin. The analogue, [Phe^3,Leu^{13}]porcine motilin (OHM-11526), displaces iodinated [Nle^{13}]porcine motilin bound to a homogenate of rabbit antral smooth muscle tissue. The dissociation constant (pK_d) was 9.26 ± 0.04, versus 9.11 ± 0.01 for motilin and 8.24 ± 0.06 for ANQ-11125, the (1–14) fragment of OHM-11526. The Hill coefficient was close to one and Schild plot analysis confirmed the competitive nature of the interaction. In the tissue bath OHM-11526 was unable to induce contractions of segments of rabbit duodenum. At a concentration of 10^{-6} M, OHM-11526 inhibited the effect of maximally effective doses of porcine motilin and of the erythromycin derivative, EM-523, but was without effect on contractions induced by acetylcholine, substance P and serotonin. Increasing doses of OHM-11526 shifted the dose-response curves of motilin and EM-523 to the right, but caused a depression of the maximal response as well. From the motilin curves, and assuming a dual competitive and non-competitive interaction, the pA_2 was 7.79 ± 0.08, the pD'_2 6.91 ± 0.08. The EM-523 curves yielded comparable data ($pA_2 = 8.10 \pm 0.12$ and $pD'_2 = 7.06 \pm 0.13$). OHM-11526 also blocked the motilin responses observed with smooth muscle strips from the rabbit and human antrum. However, in a preparation of the chicken small intestine, OHM-11526 was a full agonist with a potency ($pD_2 = 6.84$) comparable to that of porcine motilin ($pD_2 = 6.71$). Our data confirm the interaction of motilides with the motilin receptor. Due to its increased affinity for the motilin receptor, OHM-11526 will be a valuable tool for studying the physiology of motilin and the pharmacology of motilin and motilides.

Keywords: Motilin; Motilin receptor antagonist; Receptor binding; Smooth muscle contraction; Erythromycin; Motilide

1. Introduction

Motilin is a peptide of 22 amino acids which induces contractions in smooth muscle preparations of the antrum and duodenum from man, rabbit and cat, and also in preparations of the rabbit colon, in vitro (Adachi et al., 1981; Bormans et al., 1986; Depoortere et al., 1991, 1993a; Peeters et al., 1988; Strunz et al., 1975). Motilin has been isolated and purified from the duodenal mucosa of hog (Brown et al., 1973), dog (Poitras et al., 1983), rabbit (Banfield et al., 1992) and cat (Depoortere et al., 1993b). Human motilin has recently been isolated from a tumor, and is identical to porcine motilin (De Clercq et al., 1995). Binding studies showed a motilin receptor to be present in antral and duodenal smooth muscle tissue of rabbit (Bormans et al., 1986), man (Peeters et al., 1988) and cat (Depoortere et al., 1993a). This receptor belongs to the family of G protein-coupled receptors (Depoortere and Peeters, 1995).

In vivo, motilin appears to be involved, in man and in dog, in the induction of a motility pattern originating in the stomach and migrating distally which is known as phase 3 of the migrating motor complex. Motilin induces this pattern (Itoh et al., 1975; Vantrappen et al., 1979) and motilin plasma levels fluctuate in accordance with its occurrence (Poitras et al., 1980; Peeters et al., 1980). Nevertheless the physiological role of motilin remains uncertain (Sarna, 1985), also because motilin's mechanism of action in vivo has not been clarified. While pharmacological studies indicate that in vitro contractility is due to interaction with smooth muscle motilin receptors, in vivo motilin acts through cholinergic, opiate and serotonergic pathways (Fox, 1990; Itoh et al., 1991).

Recent studies have shown that the macrolide antibiotic, erythromycin A, mimics the effect of motilin in

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vivo on the motility pattern in the fasted state (Itoh et al., 1984). Based on in vitro studies we have claimed that erythromycin is a motilin agonist (Peeters et al., 1989; Peeters, 1993). However, the role of the motilin receptor in mediating motility induced by motilides in vivo has not been elucidated.

Motilin receptor antagonists could be useful to solve these issues. Recently, the first selective motilin antagonist has been described, but its affinity for the motilin receptor was about 10 times lower than the affinity of motilin itself (Peeters et al., 1994). Based upon structure-activity studies we expected that [Phe^3,Leu^{13}]-porcine motilin would be a more potent motilin antagonist and the pharmacological profile of this compound is described in this paper.

2. Materials and methods

2.1. Materials

EM-523 (de(*N*-methyl)-*N*-ethyl-8,9 anhydroerythromycin A 6,9-hemiacetal) developed by Dr. Omura of the Kitasato Institute (Tokyo, Japan) was a gift from Takeda Chemical Industries (Osaka, Japan). [Phe^3,Leu^{13}]Porcine motilin-(1–14) (ANQ-11125) and [Phe^3,Leu^{13}]porcine motilin (OHM-111526) (patents applied for) were synthesized by Ohmeda (Ohmeda, Murray Hill, NJ, USA). The *nor*-leucine^{13} analogue of porcine motilin ([Nle^{13}]porcine motilin) was purchased from Novabiochem (Läufelfingen, Switzerland).

2.2. Methods

Binding studies

Binding studies were carried out on crude homogenates prepared from rabbit antrum as described previously (Bormans et al., 1986). Briefly, membranes were incubated with ^{125}I-[Nle^{13}]porcine motilin (specific activity: ±1500 cpm/fmol, final concentration 50 pM) in 50 mM Tris-buffer (1.5% bovine serum albumin, 10 mM $MgCl_2$, pH 8.0) at 30°C for 60 min. The reaction was stopped by adding cold buffer and membrane-bound motilin was separated by centrifugation at 1000 $\times g$. All data were corrected for non-specific binding determined after the addition of an excess of [Nle^{13}]porcine motilin. Displacement curves were obtained by adding increasing amounts of OHM-11526, [Nle^{13}]porcine motilin or ANQ-11125 to the incubation media. In order to construct Schild plots, binding was also measured with different label concentrations (18–178 pM).

Contraction studies

The biological activity of OHM-11526 was tested in a tissue bath by measuring isotonically its contractile effect on segments of rabbit or chicken duodenum suspended in the longitudinal direction, or on strips prepared from the human antrum. The methodology has been described in an earlier report (Depoortere et al., 1990).

The effect of OHM-11526 upon the contractile response to maximally effective doses of motilin (10^{-7} M), acetylcholine (10^{-4} M), substance P (10^{-6} M), erythromycin A (10^{-5} M), EM-523 (10^{-6} M) or 5-hydroxytryptamine (10^{-5} M) was studied by incubating tissues for 10 min with 10^{-6} M OHM-11526 before challenging them with the respective agonist. Schild plots were constructed from experiments in which duodenal segments were incubated with different concentrations of the antagonist for 10 min before establishing a cumulative dose-response curve for motilin or EM-523. Responses were always expressed relative to the maximum obtained with acetylcholine (10^{-4} M).

Data analysis

Displacement curves were fitted by the non-linear least-squares procedure of the SAS software program (SAS Institute, Cary, NC, USA). The dissociation constant (K_d) was derived from the curve-fitting procedure, and also from the Schild plot by regression analysis.

An estimate of the pA_2 value of the antagonist in the contraction studies was obtained from regression analysis of the Schild plot.

However, because contraction dose-response curves in the presence of increasing concentrations of antagonist showed not only a shift of the curves but were also accompanied by a depression of the maximal response, the data were also analysed using the model for dual antagonists as detailed by Van den Brink (1977).

3. Results

3.1. Binding experiments

Motilin, ANQ-11125 and OHM-11526, displaced ^{125}I-[Nle^{13}]porcine motilin bound to smooth muscle membranes prepared from the antrum of the rabbit (Fig. 1). The dissociation constant (pK_d) derived from the displacement curve was 9.26 ± 0.04 ($n = 5$) for OHM-11526 compared to 8.24 ± 0.06 ($n = 4$) for ANQ-11125. The latter value is in good agreement with the result from a previous study where the pK_d of ANQ-11125 was calculated to be 8.16 ± 0.10. From a large number of motilin displacement curves, a historical value of 9.11 ± 0.01 ($n = 120$) has been derived for the pK_d of this peptide. The pK_d of OHM-11526 is slightly but significantly higher than the pK_d of motilin ($P < 0.05$), and about one log unit higher than the pK_d of ANQ-11125 ($P < 0.001$).

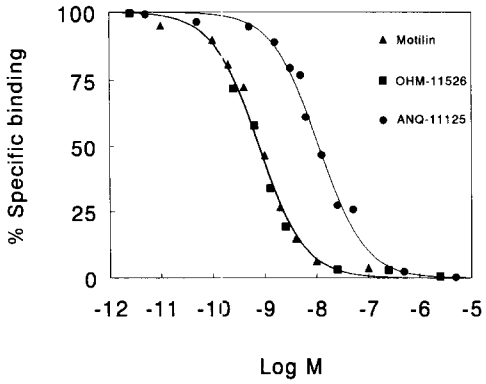

Fig. 1. Displacement of iodinated [Nle^{13}]porcine motilin bound to a membrane preparation of rabbit antral smooth muscle tissue by unlabeled [Nle^{13}]porcine motilin (▲), OHM-11526 (■) and ANQ-11125 (●).

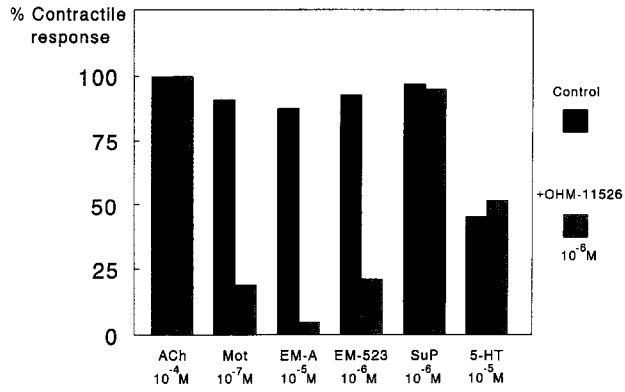

Fig. 2. Contractile response of rabbit duodenal segments to motilin (10^{-7} M), erythromycin A (10^{-5} M), EM-523 (10^{-6} M), acetylcholine (10^{-4} M), substance P (10^{-6} M) and 5-hydroxytryptamine (10^{-5} M) under control conditions (the response towards 10^{-4} M acetylcholine is taken as 100%) and in the presence of 10^{-6} M of OHM-11526. Means of four experiments.

The Hill coefficient was close to unity for OHM-11526 (0.95 ± 0.03), ANQ-11125 (1.11 ± 0.01) and motilin (1.0 ± 0.08), showing that the displacement is of a competitive type at a single set of binding sites. Schild plot analysis confirmed the competitive nature of the interaction. The line which was fitted through the motilin data had a slope of 0.91 ± 0.07, against 1.08 ± 0.06 for OHM-11526 and 0.89 ± 0.06 for ANQ-11125. In no case did the slope differ significantly from 1.0.

3.2. Contractility measurements

OHM-11526 at concentrations between 1 nM and 1 μM did not contract the antral strips or duodenal segments. Together with the binding data, these results indicated that OHM-11526 was an antagonist. Therefore the effect of 10^{-6} M OHM-11526 on the contraction induced by maximally effective doses of motilin (10^{-7} M), erythromycin A (10^{-5} M), EM-523 (10^{-6} M), acetylcholine (10^{-4} M), substance P (10^{-6} M) or 5-hydroxytryptamine (10^{-5} M) on rabbit duodenal segments was investigated (Fig. 2). OHM-11526 reduced the response towards motilin to 22 ± 7% (control 91 ± 10%), erythromycin A to 5 ± 2% (control 88 ± 8%) and EM-523 to 24 ± 8% (control 93 ± 5%), but had no effect on the response to acetylcholine, substance P or 5-hydroxytryptamine.

The effect on motilin-and EM-523-induced contractility in the duodenum was studied in more detail by recording dose-response curves in the presence of increasing concentrations of OHM-11526. A selection of some of the data is shown in Fig. 3. OHM-11526 caused a rightward shift of the dose-response curves to EM-523 and motilin, and the shift became more pronounced with increasing concentrations of OHM-11526. In the presence of $10^{-7.5}$ M OHM-11526 the pD_2 value for motilin was shifted from 8.18 ± 0.07 (control)

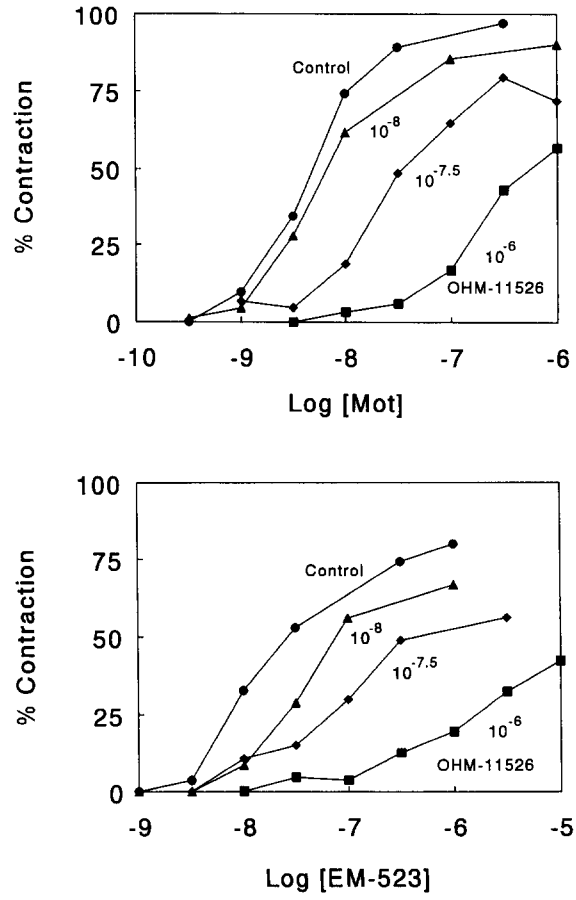

Fig. 3. Dose response curves of [Nle^{13}]porcine motilin (top tracing) and of EM-523 (bottom tracing) recorded isotonically with a segment of rabbit duodenum in the presence of different concentrations of OHM-11526. Curves were recorded under control conditions (●) or after a 10-min equilibration period with OHM-11526 at concentrations of 10^{-8} M (▲), $10^{-7.5}$ M (◆) and 10^{-6} M (■).

to 7.36 ± 0.04 and for EM-523 from 7.72 ± 0.07 (control) to 7.10 ± 0.01. The Schild plots yielded pA_2 values of 8.07 (motilin data) and 8.21 (EM-523), confirming that OHM-11526 was a more potent antagonist than ANQ-11125. Indeed the values for this compound were 7.03 (motilin data) and 7.55 (EM-523 data) (Peeters et al., 1994).

However, the validity of this analysis may be questioned, as the slope of the Schild plot was not 1.0. Hill plot analysis also indicated that the shift of the dose-response curves was not parallel. The slope of the motilin curves decreased from 1.01 ± 0.04 (control) to 0.42 ± 0.04 (in the presence of 10^{-6} M OHM-11526), and for the EM-523 curves from 0.87 ± 0.09 to 0.63 ± 0.08.

Besides the shift of the dose-response curves, a depression of the maximal contractile response became apparent with increasing doses of antagonist, as can be seen in Fig. 3. This profile resembles the results obtained with unsurmountable antagonists. The pA_2 value for the competitive interaction and the pD'_2 value of

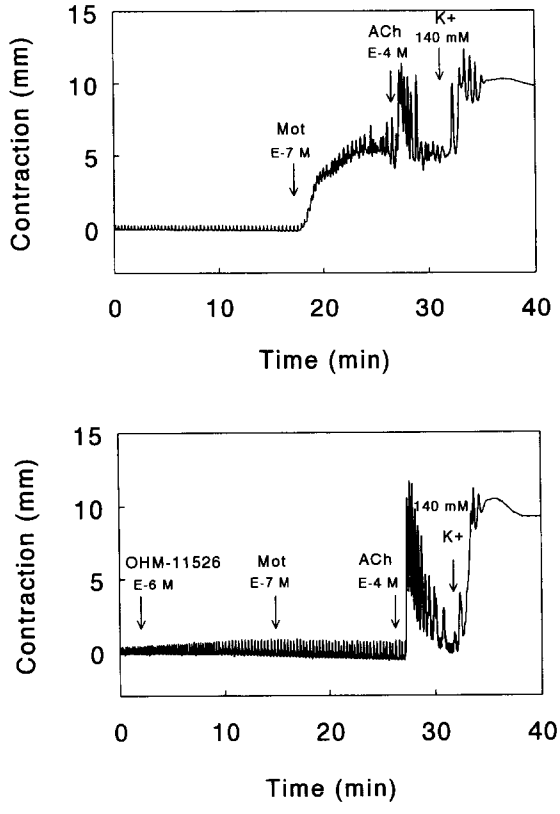

Fig. 4. Effect of OHM-11526 on the contractile response of human antral strips to maximal effective doses of [Nle^{13}]porcine motilin. Top tracings show the control response to [Nle^{13}]porcine motilin (10^{-7} M) followed by stimulation with acetylcholine (10^{-4} M) and K^+ (140 mM). Bottom tracings show the response of an adjacent strip first incubated for 10 min with OHM-11526 (10^{-6} M), prior to challenge with [Nle^{13}]porcine motilin (10^{-7} M), acetylcholine (10^{-4} M) or K^+ (140 mM).

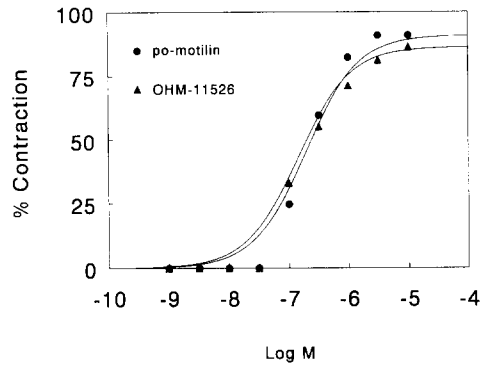

Fig. 5. Comparison of the contractile response to [Nle^{13}]porcine motilin and OHM-11526 on duodenal segments of the chicken. The compounds were added cumulatively to the tissue bath and the response was expressed relative to the maximum obtained with acetylcholine (10^{-4} M).

the metactoid interaction was therefore calculated using the method of Van den Brink (1977). This led to pA_2 values of 7.79 ± 0.08 ($n = 23$) from the motilin curves and 8.10 ± 0.12 ($n = 18$) from the EM-523 curves. These values correspond well with those calculated from the Schild plot. The magnitude of the pD'_2 value for the metactoid interaction was approximately 10 times smaller in both instances: 6.91 ± 0.08 ($n = 15$, motilin curves) and 7.06 ± 0.13 ($n = 14$, EM-523 curves).

To investigate if desensitization by motilin or EM-523 could be responsible for the decrease in the maximal response, dose-response curves for motilin and EM-523 in the absence and presence of 10^{-6} M OHM-11526 were obtained in a non-cumulative and a cumulative manner. The extent of the shift and the depression of the maximum of the dose-response curves were the same in both instances (data not shown).

OHM-11526 also antagonized the effect of motilin on human antral strips (Fig. 4). Towards duodenal segments of the chicken, however, OHM-11526 behaved as an agonist ($pD_2 = 6.84$) with effects comparable to those of porcine motilin ($pD_2 = 6.71$) (Fig. 5).

4. Discussion

The earliest reported antagonists of gastrointestinal peptides were cyclic nucleotides such as dibutyryl cyclic GMP or amino acid derivatives such as proglumide, weak antagonists of gastrin/CCK receptors. Since then a variety of antagonists have been developed, including non-peptide mimetics, interacting with receptors for cholecystokinin, gastrin, vasoactive intestinal peptide, secretin, gastrin-releasing peptide, neuromedin B, neurokinin, calcitonin gene-related peptide and somatostatin (Presti and Gardner, 1993). These antagonists have been useful for classifying the receptors of these

peptides and elucidating the complex regulatory mechanisms of gastrointestinal function, while some also have therapeutic potential.

On the other hand, there are some gastrointestinal peptides for which there are currently no known receptor antagonists. These peptides include enteroglucagon, glicentin, gastric inhibitory peptide, neurotensin, oxyntomodulin, pancreatic polypetide, pancreastatin, peptide YY and, until recently, motilin.

Peeters et al. (1994) recently reported the discovery of the first motilin antagonist, ANQ-11125, the Phe^3,Leu^{13} analogue of the (1–14) fragment of porcine motilin. However, the affinity of this compound for the motilin receptor is about 10 times less than the affinity of motilin itself. Because a comparable decrease of affinity and of agonist activity is present in the (1–14) fragment of the Leu^{13} analogue compared with the full-length peptide, (Macielag et al., 1992) the compound studied in this report was synthetized. OHM-11526 is the Phe^3,Leu^{13} analogue of the full-length porcine motilin molecule. As is demonstrated by our data this compound has an affinity for the motilin receptor comparable to that of motilin itself. The antagonistic properties are retained and, compared with those of ANQ-11125, enhanced. Like ANQ-11125, OHM-11526 is a selective motilin antagonist because the responses to acetylcholine, substance P and serotonin were not changed. Our data further confirm that erythromycin and its dervatives are motilin agonists in the rabbit and in humans in vitro.

However, contraction studies with motilin or EM-523 in the presence of the antagonist showed that increasing concentrations of these substances could not overcome the blocking effect of OHM-11526. A first possible explanation is that the antagonist acts as a purely metactoid antagonist on an agonistic system with a receptor reserve. At low doses of antagonist this interaction results in a shift of the dose-response curves without depression of the maximal response; at high concentrations the antagonist then inhibits the maximum response in a dose-dependent manner but without further increasing the EC_{50} value. In the present study this hypothesis can be ruled out because the criterion for the existence of spare receptors, an EC_{50} value smaller than the K_d value, is not fulfilled. Furthermore, the concentration-effect curves show that the EC_{50} value is still increasing when the maximal response is decreasing.

A second possibility is that the depression of the maximal response is due to desensitization. However, dose-response curves for motilin or EM-523 recorded in a cumulative or non-cumulative manner in the presence of a fixed concentration of antagonist gave similar results, showing that desensitization did not occur under our experimental conditions.

A third possibility is that OHM-11526 behaves as an unsurmountable antagonist. A similar mechanism of action has been described for the action of two antagonists, lysergic acid diethylamide and 2-bromo-lysergic acid diethylamide, on serotonin-elicited contractions in smooth muscle preparations (Kaumann, 1989; Xu and Purdy, 1989). An allosteric (Kaumann and Frenken, 1985) and a kinetic model (Bond et al., 1989) have been invoked to explain these findings, but further studies are needed to determine if these models can explain our results.

The only differences between porcine motilin and OHM-11526 are the substitution of Met^{13} by leucine, a substitution which has been known for long to be without effect on motilin's biological activity (Wünsch, 1976), and the replacement of Pro^3 by phenylalanine. It has been observed that replacement of Pro^3 by alanine slightly increases the biological activity (Peeters et al., 1992). As the pharmacophore involves primarily the N-terminus, this indicates that proline exerts a structural constraint upon the pharmacophore which apparently decreases the affinity for the receptor. Introduction of phenylalanine in this position has probably only a minor structural effect compared with an alanine analogue, but may introduce an additional binding site, which reduces the interaction of the part of the pharmacophore involved in transduction. Detailed structure-activity studies will be required to check this hypothesis. This will help to understand the dual effect, and may lead to the development of still better antagonists.

Our data show that OHM-11526 is also an antagonist of the human receptor, but an agonist of the avian motilin receptor. Such species-related antagonism and agonism has also been reported for other peptides. Wang et al. (1990) tested 19 des-Met carboxyl-terminal-modified bombesin analogues on bombesin-stimulated amylase secretion in guinea pig pancreatic acini. Six functioned as agonists, whereas the 13 remaining analogues functioned as antagonists. In contrast, in the rat pancreas 11 of the 19 bombesin analogues were agonists and the remaining 8, antagonists. Similar results were obtained with some short-chain bombesin pseudopeptides (Coy et al., 1990). An analogue has also been described for cholecystokinin (CCK), which is a full antagonist of the action of CCK in guinea pig pancreatic acini, but which stimulates enzyme secretion in acini from mouse and rat pancreas (Howard et al., 1984).

The increased affinity of OHM-11526 for the motilin receptor may make this compound suitable for use in vivo. Unfortunately, an in vitro model cannot be used for the dog, the species which has been mostly used for the study of motility patterns induced by motilin and motilides (Segawa et al., 1976). Furthermore, it remains to be proven that OHM-11526 is an antagonist in the dog in vivo. Meanwhile, however, OHM-11526

will be useful as a tool to study the mechanism of action of motilin and motilides in vitro.

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