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European Journal of Pharmacology 520 (2005) 29–36

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[^{3}H]Alvimopan binding to the μ opioid receptor: Comparative binding kinetics of opioid antagonists

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Received 4 April 2005; received in revised form 2 August 2005; accepted 5 August 2005
Available online 13 September 2005

Abstract

Alvimopan is a novel peripheral μ opioid antagonist in clinical development for the management of post-operative ileus and opioid-induced bowel dysfunction. We hypothesized that the long duration of action of alvimopan might be related to a slower dissociation rate from the μ opioid receptor compared to other shorter acting antagonists. The dissociation rate of alvimopan from the μ opioid receptor ($t_{1/2}$=30–44 min) was comparable to that of the long acting partial agonist buprenorphine ($t_{1/2}$=44 min), but was slower than those of the antagonists naloxone ($t_{1/2}$=0.82 min) and *N*-methylnaltrexone ($t_{1/2}$=0.46 min). Also, increases in the apparent affinities and potencies of buprenorphine and alvimopan, but not of naloxone and methylnaltrexone, were observed upon preincubation with the μ opioid receptor. Consistent with its long duration of action, alvimopan has a slow dissociation rate from the μ opioid receptor compared to other shorter acting antagonists and may be more potent if administered prior to dosing with exogenous opioids.

Keywords: Alvimopan; *N*-methylnaltrexone; Opioid receptor; Opioid antagonist; Kinetics

1. Introduction

Opioid-induced slowing of gastrointestinal transit results in constipation and other debilitating side effects that limit the use of opioid therapy in chronic pain (Reisine and Pasternak, 1996). Initial efforts to reverse the gastrointestinal effects of opioids were made using the opioid antagonist naloxone (Kreek et al., 1983; Culpepper-Morgan et al., 1992), but its therapeutic utility was limited due to concurrent antagonism of opioid analgesia. Later, quaternized derivatives of opioid antagonists resulted in greater peripheral selectivity (Brown and Goldberg, 1985). In particular, clinical studies with *N*-methylnaltrexone, the quaternized derivative of naltrexone, have shown that it is effective in reversing opioid-induced slowing of the gastrointestinal tract without antagonizing analgesia (Yuan et al., 1996, 2000). In addition, clinical studies with alvimopan (formerly ADL 8-2698 and LY246736), [[2(*S*)-[[4(*R*)-(3-hydroxyphenyl)-3(*R*),4-dimethyl-1-piperidinyl] methyl]-1-oxo-3-phenylpropyl]amino]acetic acid dihydrate (Fig. 1), a novel peripherally acting opioid antagonist, have demonstrated that it, like *N*-methylnaltrexone, is effective in reversing opioid-induced slowing of the gastrointestinal tract without antagonizing analgesia (Paulson et al., 2005; Fricke et al., 2000). Furthermore, clinical trials have also shown that alvimopan shortens the time to recovery for postoperative ileus, an impairment of gastrointestinal function that often delays hospital discharge for abdominal surgery patients (Delaney et al., 2005; Wolff et al., 2004; Schmidt, 2001; Taguchi et al., 2001). Like *N*-methylnaltrexone, alvimopan has limited intestinal absorption and low oral bioavailability, and hence acts selectively in the gastrointestinal tract after oral administration to antagonize peripheral opioid receptors, thereby stimulating gut motility and secretion (Zimmerman et al., 1994a,b; Schmidt, 2001; Foss, 2001).

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doi:10.1016/j.ejphar.2005.08.008

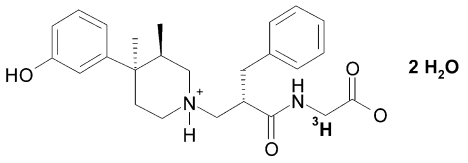

Fig. 1. Structure of [^{3}H]alvimopan (specific activity 28 Ci/mmol).

In vivo studies in mice comparing alvimopan and *N*-methylnaltrexone have shown that alvimopan is 200-fold more potent at antagonizing the inhibitory effects of morphine on gastrointestinal transit and secretion (DeHaven et al., in preparation; Little et al., 2001). In addition, alvimopan has a long duration of action (Long et al., 1999; Zimmerman et al., 1994b), and it is effective at antagonizing the inhibitory effects of morphine on gastrointestinal transit and secretion for four times longer than *N*-methylnaltrexone (DeHaven et al., in preparation). We hypothesized that the binding kinetics of these drugs at the μ opioid receptor might provide some insight into the observed differences in their durations of action. In order to test this hypothesis, we characterized [^{3}H]alvimopan binding to the cloned human μ opioid receptor and compared its binding kinetics to those of *N*-methylnaltrexone and other opioid antagonists.

2. Materials and methods

2.1. Materials

[^{3}H]Diprenorphine (40 Ci/mmol), custom labeled [^{3}H] alvimopan (28 Ci/mmol; 38.5 Ci/mmol), and [^{35}S]GTPγS (1250 Ci/mmol) were obtained from PerkinElmer Life Sciences Inc. (Boston, MA). All compounds tested were obtained from Sigma-Aldrich (St. Louis, MO) with the exception of *N*-methylnaltrexone, which was obtained from Mallinckrodt Chemical Inc. (St. Louis, MO). The expression vector containing the cloned human μ opioid receptor was obtained from Ohmeda Pharmaceutical Products Division (Murray Hill, NJ). Cells expressing the receptor were grown and harvested at Adolor Corporation.

2.2. Preparation of membranes for μ opioid receptor binding

CHO-K1 cells were cultured in Ham′s F-12 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin under 5% CO_2 atmosphere. Cells were transfected with plasmids containing the μ opioid receptor cDNA using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) followed by selection of the transfected cells with 1.0 mg/ml G418 (Mediatech, Herndon, VA). Cell foci resistant to G418 were characterized with respect to binding of [^{3}H]diprenorphine. Cells stably expressing the cloned human μ opioid receptor were harvested by scraping them from the culture flask and centrifuging them at 1000 ×*g* for 10 min. The cells were resuspended in assay buffer (50 mM Tris HCl, pH 7.8, 1.0 mM EGTA, 5.0 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 200 μg/ml bacitracin, 0.5 μg/ml aprotinin) and centrifuged as before. The resulting pellet was resuspended in assay buffer and homogenized using a Polytron homogenizer (Brinkmann, Westbury, NJ) at setting 1 for 30 s. The homogenate was then centrifuged at 48,000 ×*g* for 10 min at 4 °C and the pellet was resuspended in assay buffer at 1 mg of protein/ml and aliquots stored at −80 °C until used.

2.3. Saturation binding of [^{3}H]alvimopan to the μ opioid receptor

After dilution in assay buffer and re-homogenization, membrane preparation (20 μg of protein/well) was added to assay mixtures containing a series of concentrations of [^{3}H] alvimopan ranging from 0.012 to 14 nM in a final volume of 0.5 ml of assay buffer. Nonspecific binding was determined in the presence of 10 μM naloxone at each concentration of [^{3}H]alvimopan and was less than 15% of total binding. After incubation at room temperature for 2 h, the assay was filtered through GF/B filters that had been presoaked in a solution of 0.5% (w/v) polyethylenimine and 0.1% (w/v) bovine serum albumin in water. The filters were washed four times with 1 ml of ice-cold 50 mM Tris HCl (pH 7.8). Microscint 20 (PerkinElmer Life Sciences Inc., Boston, MA) (30 μl) was added to each sample and the radioactivity remaining on the filters was determined with a TopCount microplate scintillation counter (PerkinElmer Life Sciences Inc., Boston, MA). The K_d and B_{max} values were obtained from nonlinear regression fits of the data to a one-site binding model using GraphPad Prism® version 4.02 for Windows (GraphPad Software, San Diego, CA). Preliminary experiments demonstrated that equilibrium binding was reached by 90 min and was stable for at least an additional 90 min, that the binding was linear with protein concentration in the range of concentrations tested, and that the filter wash protocol minimized nonspecific binding without affecting specific binding.

2.4. Association rate of [^{3}H]alvimopan binding to the μ opioid receptor

Membrane preparation (20 μg of protein/well) was added to assay mixtures containing concentrations of [^{3}H]alvimopan ranging from 0.30 to 4.0 nM in assay buffer and specific binding was determined after various times as described above. Nonspecific binding was determined in the presence of 10 μM naloxone at each time point. The observed association rate constant (k_{obs}) at each concentration of [^{3}H] alvimopan was determined from a best nonlinear regression fit of data to a one-phase exponential association model using GraphPad Prism® version 4.02 for Windows. Plotting the k_{obs} values versus the concentration of [^{3}H]alvimopan resulted in a straight line with a slope equal to the association rate

constant (k_{on}) and a *Y*-intercept equal to the dissociation rate constant (k_{off}).

2.5. Rate of dissociation of [^{3}H]alvimopan-m opioid receptor complex

Mixtures of membrane preparation (20 μg of protein/well) and [^{3}H]alvimopan (0.5 to 1 nM) in a final volume of 300 μl of assay buffer were incubated for 90 min at room temperature until equilibrium binding was reached. Excess unlabeled naloxone (10 μM) was then added to each assay to bring the total volume to 0.5 ml and the incubation was continued for various times before termination by filtration as described above. The k_{off} values were obtained from nonlinear regression fits of the data to a one-phase exponential decay model using GraphPad Prism® version 4.02 for Windows.

2.6. Inhibition of μ opioid receptor binding by test compounds

Assays to measure inhibition of binding to the cloned human μ opioid receptor contained [^{3}H]diprenorphine (0.4 to 1 nM) or [^{3}H]alvimopan (0.86 to 1.1 nM), test compound at concentrations ranging from 36 pM to 10 μM, and membrane preparation (20 μg of protein/well) in a total volume of 0.5 ml of assay buffer. After incubation at room temperature for 90 min, assays were terminated and the bound radioactivity determined as described above. The K_i values were determined by Cheng–Prusoff corrections of IC_{50} values derived from nonlinear regression fits of the data to a one-site competition model using GraphPad Prism® version 4.02 for Windows.

2.7. Association and dissociation rates of unlabeled compounds

Membrane preparation (100 μg/well) was added to assay mixtures containing [^{3}H]alvimopan (0.50–0.77 nM) in the presence and absence of test compounds in assay buffer and the specific binding determined at various times as described above. Nonspecific binding was determined in the presence of 10 μM naloxone at the latest time point. The concentration of test compound was equal to approximately four times its K_i value obtained in equilibrium experiments. This concentration was not sufficient to completely inhibit specific binding at equilibrium. Data were analyzed according to the equations described by Motulsky and Mahan (1984),

$$R + L \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{RL} \quad R + I \underset{k_4}{\overset{k_3}{\rightleftarrows}} \mathrm{RI}$$

$$[\mathrm{RL}] = \frac{Nk_1[L]}{K_F - K_S}\left[\frac{k_4(K_F - K_S)}{K_F K_S} + \frac{(k_4 - K_F)}{K_F}\exp(-K_F t) - \frac{(k_4 - K_S)}{K_S}\exp(-K_S t)\right]$$

where,

$$K_F = 0.5\left[\left(K_A + K_B + \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I]}\right)\right],$$

$$K_S = 0.5\left[\left(K_A + K_B - \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I]}\right)\right],$$

$$K_A = k_1[L] + k_2, K_B = k_3[I]k_4,$$

and $[L]$ = free radioligand concentration; $[I]$ = free competitor concentration; R = free receptors; [RL] = radioligand specific binding; RI = receptor competitor complex; $N = R + \mathrm{RL} + \mathrm{RI}$ = total number of receptors, i.e. B_{max} (pmol/mg protein); k_1, k_2 and k_3, k_4 are the radioligand and competitor association (k_{on}) and dissociation (k_{off}) rate constants, respectively. The k_1, k_2, and B_{max} values for [^{3}H]alvimopan binding were determined in separate experiments. For this membrane preparation, the B_{max} value for [^{3}H]alvimopan binding was 0.34 pmol/mg protein. GraphPad Prism® version 4.02 for Windows was used to solve for k_3 and k_4.

2.8. [^{3}H]Diprenorphine binding to the μ opioid receptor preincubated with alvimopan, buprenorphine, naloxone, or N-methylnaltrexone

Assays measuring the inhibition of [^{3}H]diprenorphine binding to the cloned human μ opioid receptor were performed as described above. A series of twelve serial dilutions of test compound ranging from 3.2 pM to 10 μM were preincubated with the μ opioid receptor for various times, ranging from 0 to 60 min prior to the addition of [^{3}H]diprenorphine. After an additional incubation at room temperature for 90 min, assays were terminated and the bound radioactivity determined as described above. The K_i values were determined by Cheng–Prusoff corrections of IC_{50} values as described above.

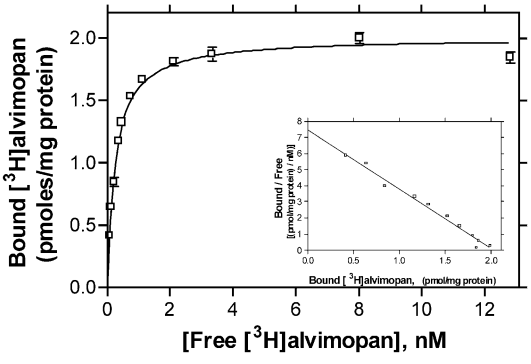

Fig. 2. Saturation isotherm of [^{3}H]alvimopan binding to cloned human μ opioid receptors. Membranes prepared from CHO cells expressing the cloned human μ opioid receptor were incubated with a series of concentrations of [^{3}H]alvimopan as described in Materials and methods. *Inset*: Scatchard plot. Data shown are the means ± S.E.M. of triplicate values from a single experiment that was repeated three times with similar results. Where not shown, error bars are contained within the symbols.

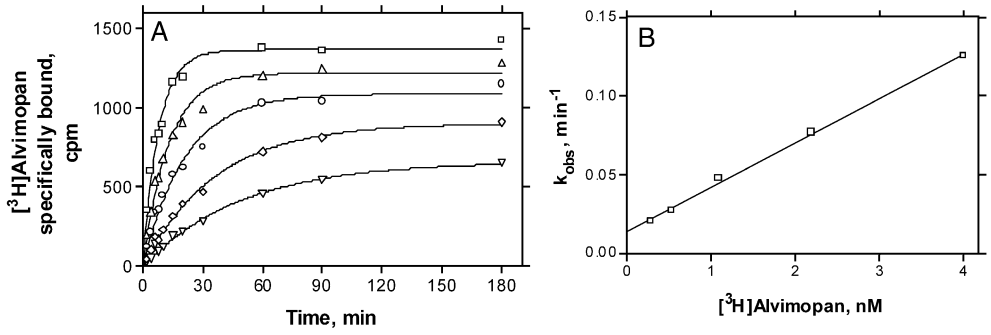

Fig. 3. Association rate of [^{3}H]alvimopan binding to cloned human μ opioid receptors. Membranes prepared from CHO cells expressing the cloned human μ opioid receptor were incubated for various times with a series of concentrations of [^{3}H]alvimopan as described in Materials and methods. (A) Specific binding over time with [^{3}H]alvimopan at 0.30 nM (▽), 0.53 nM (◇), 1.1 nM (○), 2.2 nM (△), 4.0 nM (□); (B) Observed association rate constants (k_{obs}) at different concentrations of [^{3}H]alvimopan. Data shown are individual values from a single experiment that was repeated three times with similar results.

2.9. μ Opioid receptor-mediated [^{35}S]GTPγS binding in membrane preparations preincubated with alvimopan, buprenorphine, naloxone or N-methylnaltrexone

A series of concentrations of alvimopan, buprenorphine, naloxone or *N*-methylnaltrexone as described above were added to assay mixtures containing membrane preparation (50–100 μg/well), 3.0 μM GDP, 75 mM NaCl, 10 mM $MgCl_2$, and 2.1 mM dithiothreitol in 96-well basic Flashplates® (PerkinElmer Life Sciences, Boston, MA). Either immediately after the addition of test compound or after a 60 min preincubation, [^{35}S]GTPγS (80–100 pM) and loperamide (100 nM) were added to each well and the assay was incubated for an additional 60 min at room temperature. This concentration of loperamide was previously established as sufficient to stimulate [^{35}S]GTPγS binding to approximately 80% of the maximum for loperamide. After incubation, the plates were sealed, centrifuged at 800 ×*g* in a swinging bucket rotor for 5 min and bound radioactivity was determined with a TopCount microplate scintillation counter. IC_{50} values were derived from nonlinear regression fits of the data to a one-site competition model using GraphPad Prism® version 4.02 for Windows.

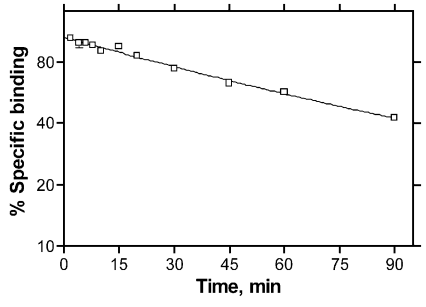

Fig. 4. Dissociation of [^{3}H]alvimopan from the cloned human μ opioid receptor. Membranes prepared from CHO cells expressing the cloned human μ opioid receptor were incubated with [^{3}H]alvimopan until equilibrium was reached, followed by the addition of 10 μM unlabeled naloxone and subsequent incubation for various times as described in Materials and methods. Data shown are the means±S.E.M. of triplicate values from a single experiment that was repeated three times with similar results. Where not shown, the error bars are contained within the symbols. The line was obtained from a nonlinear regression fit of the data to a one-phase exponential decay model using GraphPad Prism® version 4.02 for Windows.

3. Results

3.1. [^{3}H]Alvimopan binding to the cloned human μ opioid receptor

Saturation binding experiments showed that [^{3}H]alvimopan bound with a high affinity (K_d=0.35 nM, 95% CI: 0.23–0.54) and a B_{max} value of 2.1±0.12 pmol/mg protein to membrane preparations derived from CHO cells expressing

Table 1
Affinities of opioid agonists and antagonists determined with either [^{3}H]alvimopan or [^{3}H]diprenorphine as the competing radioligand at the cloned human μ opioid receptor

	K_i (nM)	
	[^{3}H]Alvimopan	[^{3}H]Diprenorphine
Antagonists		
Buprenorphine	0.74 (0.55–1.0)	0.52 (0.41–0.67)
Naltrexone	0.86 (0.61–1.2)	1.1 (0.74–1.5)
Nalmefene	1.0 (0.78–1.2)	0.67 (0.57–0.78)
Naloxone	5.4 (3.5–8.3)	3.3 (2.7–4.1)
N-methylnaltrexone	16 (10–24)	26 (17–39)
N-methylnaloxone	420 (160–1100)	140 (52–380)
Agonists		
Loperamide	3.8 (2.2–6.7)	2.6 (2.1–3.2)
Methadone	24 (15–36)	14 (9.4–21)
Morphine	28 (16–50)	20 (13–31)
Fentanyl	34 (20–57)	14 (6.6–30)
BW373U86	170 (100–310)	92 (76–110)
U50,488H	>1000	>1000

Compounds were titrated in [^{3}H]alvimopan and [^{3}H]diprenorphine binding assays with μ opioid receptor membrane preparations and the K_i values determined as described in Materials and methods. Values shown are the geometric means of the K_i values and 95% confidence intervals of 3 to 23 determinations.

Table 2
Association and dissociation rate constants of antagonists at the cloned human μ opioid receptor

	k_{on} ($\mu mol^{-1} min^{-1}$)	k_{off} (min^{-1})	$t_{1/2}$ (min)[a]	K_i (nM)[b]
Alvimopan	39±27	0.023±0.0099	30	0.60
Buprenorphine	26±13	0.016±0.0086	44	0.61
Naltrexone	100±57	0.35±0.091	2.0	3.5
Nalmefene	58±23	0.29±0.10	2.4	5.0
Naloxone	47±21	0.85±0.33	0.82	18
N-methylnaltrexone	13±4.4	1.5±0.44	0.46	110
N-methylnaloxone	2.5±1.2	1.2±0.72	0.58	480

Association of [^{3}H]alvimopan binding to the cloned human μ opioid receptor was measured in the presence of a single concentration of antagonist and the k_{on} and k_{off} values determined as described in Materials and methods. Values shown are the means±S.E.M. of 4 to 7 determinations.
[a] Dissociation half-times ($t_{1/2}$) were calculated from $0.693/k_{off}$.
[b] K_i values were calculated from k_{off}/k_{on}.

the cloned human μ opioid receptor (Fig. 2). This K_d value was consistent with its reported K_i value of 0.44 nM as determined by displacement of [^{3}H]diprenorphine binding to the cloned human μ opioid receptor (DeHaven et al., in preparation). The association rate constant (k_{on}) of [^{3}H]alvimopan binding to the μ opioid receptor was 2.6×10^7 M^{-1} min^{-1}, and the observed association rate constants (k_{obs}) were linearly proportional to the concentration of [^{3}H]alvimopan (Fig. 3), which is consistent with binding to a single population of non-interacting sites (Limbird, 1996; Motulsky, 1999). The extrapolated dissociation rate constant (k_{off}) was 0.016 min^{-1} ($t_{1/2}$=44 min) and the calculated K_d value (k_{off}/k_{on}) was 0.62 nM, which is consistent with the K_d value obtained in saturation experiments. The dissociation of bound [^{3}H]alvimopan from the cloned μ opioid receptor was first-order (Fig. 4), as determined in the presence of excess unlabeled naloxone, with a k_{off} value of 0.012 min^{-1} ($t_{1/2}$=57 min), which is consistent with the k_{off} value extrapolated from plots of k_{obs} values versus concentration of [^{3}H]alvimopan. Results shown in Table 1 demonstrate that the potencies of various opioid agonists and antagonists were essentially the same when determined by their abilities to displace [^{3}H]alvimopan binding as when determined by their abilities to displace [^{3}H]diprenorphine binding.

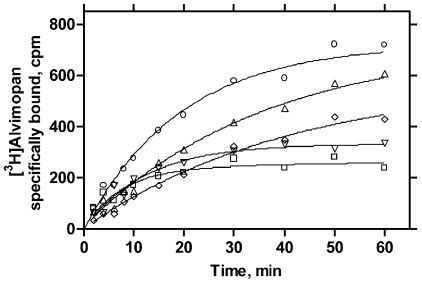

Fig. 5. Determination of association and dissociation rate constants for unlabeled alvimopan, buprenorphine, naloxone and *N*-methylnaltrexone at the cloned human μ opioid receptor. Membranes prepared from CHO cells expressing the cloned human μ opioid receptor were incubated for various times with [^{3}H]alvimopan in the absence (○) or presence of 2.0 nM alvimopan (▽), 3.0 nM buprenorphine (□), 25 nM naloxone (△), or 150 nM *N*-methylnaltrexone (◇) and the data analyzed according to the model proposed by Motulsky and Mahan (1984) as described in Materials and methods. Data shown are the means of duplicate values from a single experiment that was repeated four to seven times with similar results as summarized in Table 2.

3.2. Association and dissociation rates of unlabeled compounds

The rate of association of [^{3}H]alvimopan binding to the μ opioid receptor was measured in the absence and presence of a single concentration of unlabeled compound equal to four to five times the K_i value for the latter as determined in equilibrium binding experiments. These concentrations of unlabeled compounds were not sufficient to completely inhibit specific [^{3}H]alvimopan binding at equilibrium. Data were analyzed according to the equations described by Motulsky and Mahan (1984) for the determination of association and dissociation rate constants for a competing unlabeled ligand. Unlabeled alvimopan was included in the experiments as a control. Buprenorphine and alvimopan had the slowest dissociation rates, while *N*-methylnaltrexone had the fastest dissociation rate (Table 2, Fig. 5). The half time of buprenorphine dissociation (Table 2) was consistent with that reported by Boas and Villiger (1985) in rat brain homogenates. Naltrexone had the fastest association rate

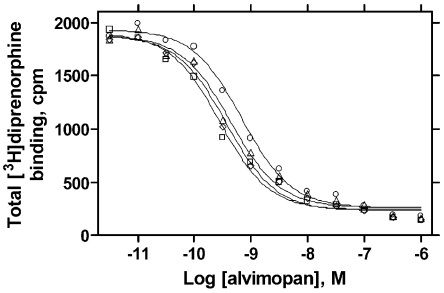

Fig. 6. Effects of preincubation on the apparent affinity of alvimopan at the cloned human μ opioid receptor. Membranes prepared from CHO cells expressing the cloned human μ opioid receptor were preincubated with a series of concentrations of alvimopan for 0 min (○), 15 min (△), 30 min (◇), or 60 min (□) prior to the addition of [^{3}H]diprenorphine, and K_i values determined as described in Materials and methods. Data shown are individual values from a single experiment that was repeated at least three times and summarized in Table 3.

Table 3
Apparent binding affinities of alvimopan, buprenorphine, naloxone, and *N*-methylnaltrexone after preincubation with the cloned human μ opioid receptor

	K_i (nM)			
Preincubation time (min)	Alvimopan	Buprenorphine	Naloxone	*N*-methylnaltrexone
0	0.32 (0.26–0.40)	0.33 (0.23–0.48)	3.2 (2.7–3.7)	22 (17–28)
15	0.19 [a] (0.13–0.26)	0.22 (0.17–0.28)	3.3 (2.3–4.7)	29 (19–43)
30	0.14 [b] (0.13–0.17)	0.17 [a] (0.14–0.20)	3.1 (3.1–4.5)	31 (22–36)
60	0.13 [b] (0.091–0.18)	0.11 [b] (0.091–0.14)	3.1 (2.4–4.1)	31 (27–37)

A series of concentrations of each compound was preincubated with membrane preparations containing the cloned human μ opioid receptor prior to the addition of [^{3}H]diprenorphine and K_i values determined as described in Materials and methods. Values shown are the geometric means and 95% confidence intervals of three to five determinations. Statistical comparisons were obtained from two-tailed, paired *t*-tests comparing results from each preincubation time with those from no preincubation.
[a] $P<0.01$.
[b] $P<0.001$.

with the μ opioid receptor, while *N*-methylnaloxone had the slowest association rate. The k_{on} and k_{off} values obtained for unlabeled alvimopan (Table 2) were consistent with those obtained using the tritiated compound (Figs. 3 and 4). In addition, the K_i value calculated from the k_{on} and k_{off} values for unlabeled alvimopan (Table 2) was consistent with its K_d value from both saturation and kinetic binding experiments, as well as its reported K_i value (0.44 nM) in [^{3}H] diprenorphine binding to the cloned human μ opioid receptor (DeHaven et al., in preparation). The K_i values calculated from the k_{on} and k_{off} values (Table 2) for the other opioid antagonists were similar to the K_i values obtained in equilibrium binding experiments, and the rank order of affinities was the same for all methods used for the determination of K_i values.

3.3. Effects of preincubation of cloned μ opioid receptor with alvimopan, buprenorphine, naloxone, or N-methylnaltrexone

Preincubation for 15 min or longer with the μ opioid receptor prior to the addition of [^{3}H]diprenorphine increased the apparent affinities of alvimopan (Fig. 6) and buprenorphine by two-to three-fold (Table 3). However, preincubation times up to 60 min did not increase the apparent affinities of naloxone or *N*-methylnaltrexone (Table 3). The apparent potencies of alvimopan (Fig. 7) and buprenorphine increased four-fold in μ opioid receptor-mediated [^{35}S]GTPγS binding after preincubation for 60 min prior to the addition of loperamide and [^{35}S]GTPγS (Table 4). However, preincubation had no effect on the apparent potencies of naloxone or *N*-methylnaltrexone in μ opioid receptor-mediated [^{35}S]GTPγS binding (Table 4).

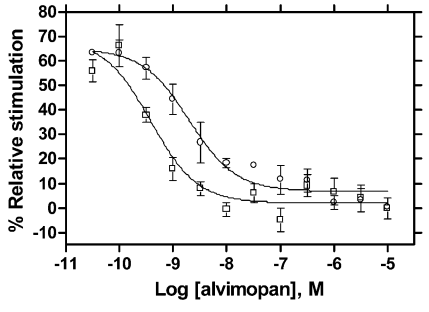

Fig. 7. Effects of preincubation on the apparent potency of alvimopan in μ opioid receptor-mediated [^{35}S]GTPγS binding. Membranes prepared from CHO cells expressing the cloned human μ opioid receptor were preincubated with a series of concentrations of alvimopan for 0 min (○) or 60 min (□) prior to the addition of 100 nM loperamide and [^{35}S]GTPγS and IC_{50} values determined a described in Materials and methods. Data represent the mean±S.E.M. of three experiments, summarized in Table 4, and are expressed as percent stimulation relative to maximal stimulation achieved with 10 μM loperamide.

4. Discussion

Alvimopan is a high affinity, competitive antagonist at the μ opioid receptor as determined by [^{3}H]diprenorphine binding, antagonism of μ opioid receptor-mediated [^{35}S]-GTPγS binding, and antagonism of μ opioid receptor-mediated inhibition of cAMP production (DeHaven et al., in preparation; Daubert et al., 2000). The present study extends these observations through characterization of [^{3}H]alvimopan binding to the cloned human μ opioid receptor. Saturation binding of [^{3}H]alvimopan to the μ opioid receptor was characterized by a linear Scatchard plot, consistent with binding to a single population of binding sites (Limbird, 1996). The rate of association of [^{3}H]alvimopan to the μ opioid receptor increased linearly with the concentration of [^{3}H]alvimopan, which is consistent with a lack of apparent cooperativity or interaction among binding sites (Motulsky, 1999; Limbird, 1996). Moreover, the dissociation rate of the [^{3}H]alvimopan-μ opioid receptor complex followed first-order kinetics, which is also consistent with reversible binding to non-interacting sites (Limbird, 1996). Similarly, the dissociation rate constant was the same regardless of whether the value was determined directly or extrapolated from the association rate experiments. In addition, determinations of the equilibrium dissociation rate constant using the association and dissociation rate constants agreed well with those obtained in equilibrium binding experiments. Therefore, we have demonstrated that [^{3}H]alvimopan is a

Table 4
Apparent functional potencies of alvimopan, buprenorphine, naloxone and *N*-methylnaltrexone after preincubation with the cloned human μ opioid receptor

	IC_{50} (nM)			
Preincubation time (min)	Alvimopan	Buprenorphine	Naloxone	*N*-methylnaltrexone
0	1.7 (0.53–5.5)	6.6 (4.7–9.8)	3.8 (2.2–6.6)	61 (24–160)
60	0.39[a] (0.28–0.53)	1.5[b] (1.3–1.6)	4.5 (3.0–6.8)	120 (41–350)

A series of concentrations of each compound was preincubated with membrane preparations containing the cloned human μ opioid receptor prior to the addition of loperamide and [^{35}S]GTPγS and the IC_{50} values determined as described in Materials and methods. Values shown are the geometric means and 95% confidence intervals of three to four determinations. Statistical comparisons were obtained from two-tailed, paired *t*-tests comparing results from preincubation to those from no preincubation.

[a] $P<0.01$.

[b] $P<0.001$.

high affinity μ opioid ligand that binds reversibly to a single population of non-interacting sites.

In the present study, we compared the association and dissociation rates of alvimopan and other opioid antagonists using the kinetics of competitive binding model proposed by Motulsky and Mahan (1984). The association and dissociation rate constants we obtained for unlabeled alvimopan and naloxone using this method were consistent with those obtained using [^{3}H]alvimopan and [^{3}H]naloxone (unpublished observations). Furthermore, the K_i value for alvimopan calculated from these parameters was similar to its K_i value in [^{3}H]diprenorphine binding (DeHaven et al., in preparation) and similar to its K_d value determined in saturation binding experiments in this study. The dissociation half-time of buprenorphine was consistent with that reported by Boas and Villiger (1985) in rat brain homogenates. In addition, the dissociation half-time of nalmefene was longer than that of naloxone, which is consistent with clinical studies using positron emission tomography scanning (Kim et al., 1997). Finally, the rank order of affinities, as determined by equilibrium binding experiments, was the same as that determined from kinetic binding experiments. Therefore, we were able to generate reliable estimates for the association and dissociation rate constants of unlabeled opioid antagonists using this model in [^{3}H]alvimopan binding to the cloned human μ opioid receptor.

The effects of opioids on gastrointestinal function are predominantly mediated by peripheral opioid receptors within the gastrointestinal tract (Tavani et al., 1980; Manara et al., 1986; Murphy et al., 1997). Therefore, the duration of action of peripherally selective opioid antagonists such as *N*-methylnaltrexone and alvimopan will be primarily governed by the rate of clearance from the gastrointestinal tract. Since only unbound drug is subject to metabolism and clearance, the slower dissociation rate of alvimopan compared to *N*-methylnaltrexone from the μ opioid receptor may, in part, explain the differences in the durations of action observed with these two drugs. It is also noteworthy that the dissociation rate of alvimopan was comparable to that of buprenorphine. It has been documented that the actions of buprenorphine extend well beyond its plasma half-life (Cowan et al., 1977; Bullingham et al., 1983). This discrepancy has been explained in part as due to the slow dissociation rate of buprenorphine from opioid receptors (Boas and Villiger, 1985). Therefore, the dissociation rates of alvimopan, buprenorphine, and *N*-methylnaltrexone are consistent with their respective durations of action, and like buprenorphine, the slow dissociation rate of alvimopan may be an important factor in its duration of action.

Studies using rat brain homogenates have demonstrated that the apparent affinity of buprenorphine is increased when it is preincubated with membrane preparations prior to the addition of [^{3}H]fentanyl, and this increase in apparent affinity is due to its slow dissociation rate (Boas and Villiger, 1985). We have confirmed and extended the observations by Boas and Villiger (1985) with respect to buprenorphine and we have demonstrated that alvimopan has a similar profile. Not only does preincubation with the μ opioid receptor result in an increase in apparent affinity for alvimopan and buprenorphine, but it also results in an increase in functional potency in antagonizing loperamide-induced stimulation of μ opioid receptor-mediated [^{35}S]GTPγS binding. For the faster dissociating μ opioid antagonists naloxone and *N*-methylnaltrexone, preincubation with the μ opioid receptor did not result in an increase in apparent affinity or potency, thereby supporting the hypothesis that the increase in affinity and potency for alvimopan and buprenorphine is due to their slow dissociation rates, rather than the experimental conditions of the assay.

This apparent increase in affinity was not the result of the combination of a slowly dissociating competitor with a rapidly dissociating radioligand since the dissociation rate constant for [^{3}H]diprenorphine was 0.020 min^{-1} ($t_{1/2}$=36 min) (unpublished observations), which is comparable to that of alvimopan and buprenorphine. We have also shown that alvimopan can completely dissociate from the μ opioid receptor after repeated washing and centrifugation of the membrane preparations (unpublished observations), suggesting that this apparent increase in affinity of alvimopan upon preincubation with the μ opioid receptor is not due to irreversible binding of alvimopan to the μ opioid receptor. Alternatively, we have shown that over the time of incubation with [^{3}H]diprenorphine (60 min) or of incubation with loperamide (60 min) only 45% of bound alvimopan will have dissociated (Fig. 4) resulting only in partial reversibility during the time frame of the experiment.

In summary, we have shown that alvimopan binds with high affinity to a single population of non-interacting sites in membrane preparations derived from CHO cells expressing the cloned human μ opioid receptor. Alvimopan has a slow rate of dissociation from the μ opioid receptor, which is comparable to that of buprenorphine and is 100-fold slower than that of *N*-methylnaltrexone. We postulate that the slow rate of dissociation of alvimopan may contribute to its long duration of action. Finally, we have shown that preincubation of alvimopan with the cloned human μ opioid receptor increases its apparent affinity at the receptor and apparent potency in a functional assay, and it may be more potent if administered prior to exogenous opioids.

References

Boas, R.A., Villiger, J.W., 1985. Clinical actions of fentanyl and buprenorphine: the significance of receptor binding. Br. J. Anaesth. 57, 192–196.

Brown, D.R., Goldberg, L.I., 1985. The use of quaternary narcotic antagonists in opiate research. Neuropharmacology 24, 181–191.

Bullingham, R.E., McQuay, H.J., Moore, R.A., 1983. Clinical pharmacokinetics of narcotic agonist–antagonist drugs. Clin. Pharmacokinet. 8, 332–343.

Cowan, A., Lewis, J.W., MacFarlane, I.E., 1977. Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. Br. J. Pharmacol. 60, 537–545.

Culpepper-Morgan, J.A., Inturrisi, C.E., Portenoy, R.K., Foley, K., Houde, R.W., Marsh, F., Kreek, M.-J., 1992. Treatment of opioid-induced constipation with oral naloxone: a pilot study. Clin. Pharmacol. Ther. 52, 90–95.

Daubert, J.D., Cassel, J.A., Guo, D., Gauntner, E.K., Kumar, V., Mansson, E., DeHaven, R.N., 2000. Inhibition of opioid receptor-mediated [35S]GTPγS binding by ADL 8-2698, a potent opioid receptor antagonist. Soc. Neurosci. Abstr. 26, 635.

DeHaven, R.N., Little, P.J., Cassel, J.A., Gottshall, S.L., Koblish, M., Long, S., Wiant, D.D., Guo, D., Mansson, E., Daubert, J.D., DeHaven-Hudkins, D., in preparation. In vitro and in vivo pharmacology of alvimopan, a novel, peripherally acting opioid antagonist.

Delaney, C.P., Weese, J.L., Hyman, N.H., Bauer, J., Techner, L., Gabriel, K., Du, W., Schmidt, W.K., Wallin, B.A., 2005. Phase III trial of alvimopan, a novel, peripherally acting, mu opioid antagonist, for postoperative ileus after major abdominal surgery. Dis. Colon Rectum 48, 1114–1129.

Fricke, J.R., Linquist, D., Carpenter, R.L., 2000. ADL 8-2698 does not antagonize opioid analgesia. Clin. Pharmacol. Ther. 67, 93.

Foss, J.F., 2001. A review of the potential role of methylnaltrexone in opioid bowel dysfunction. Am. J. Surg. 182, 19S–26S.

Kim, S., Wagner Jr., H.N., Villemagne, V.L., Kao, P.F., Dannals, R.F., Ravert, H.T., Joh, T., Dixon, R.B., Civelek, A.C., 1997. Longer occupancy of opioid receptors by nalmefene compared to naloxone as measured in vivo by a dual-detector system. J. Nucl. Med. 38, 1726–1731.

Kreek, M.-J., Schaefer, R.A., Hahn, E.F., Fishman, J., 1983. Naloxone, a specific opioid antagonist, reverses chronic idiopathic constipation. Lancet 1, 261–262.

Limbird, L.E., 1996. Cell Surface Receptors: A Short Course on Theory and Methods, second ed. Kulwer Academic Publishers, Norwell, MA.

Little, P.J., Koblish, M., DeHaven-Hudkins, D.L., Pietras, M.R., Cowan, A., 2001. ADL 8-2698, a GI restricted opioid antagonist, blocks the antisecretory and colorectal transit effects of morphine and loperamide. Soc. Neurosci. Abstr. 27, 907.2.

Long, S.L., Little, P.J., Gaul, F., Guo, D., Koblish, M., DeHaven-Hudkins, D.L., 1999. Effects of novel μ opioid antagonists on gastrointestinal (GI) transit in mice. Soc. Neurosci. Abstr. 25, 1706.

Manara, L., Bianchi, G., Ferretti, P., Tavani, A., 1986. Inhibition of gastrointestinal transit by morphine in rats results primarily from direct drug action on gut opioid sites. J. Pharmacol. Exp. Ther. 237, 945–949.

Motulsky, H.J., 1999. Analyzing Data with GraphPad Prism. GraphPad Software Inc., San Diego, CA.

Motulsky, H.J., Mahan, L.C., 1984. The kinetics of competitive radioligand binding predicted by the law of mass action. Mol. Pharmacol. 25, 1–9.

Murphy, D.B., Sutton, J.A., Prescott, L.F., Murphy, M.B., 1997. Opioid-induced delay in gastric emptying: a peripheral mechanism in humans. Anesthesiology 87, 765–770.

Paulson, D.M., Kennedy, D.T., Donovick, R.A., Carpenter, R.L., Cherubini, M., Techner, L., Du, W., Ma, Y., Schmidt, W.K., Wallin, B.A., Jackson, D., 2005. Alvimopan: an oral, peripherally acting, μ-opioid receptor antagonist for the treatment of opioid-induced bowel dysfunction — a 21 day treatment-randomized clinical trial. J. Pain 6, 184–192.

Reisine, T., Pasternak, G., 1996. Opioid analgesics and antagonists, In: Hardman, J.G., Limbird, L.E., Molinoff, P.G., Ruddon, R.W., Gilman, A.G. (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, ninth ed. McGraw-Hill, New York, pp. 521–555.

Schmidt, W.K., 2001. Alvimopan (ADL 8-2698) is a novel peripheral opioid antagonist. Am. J. Surg. 182, 27S–38S.

Taguchi, A., Sharma, N., Saleem, R.M., Sessler, D.I., Carpenter, R.L., Seyedsadr, M., Kurz, A., 2001. Selective postoperative inhibition of gastrointestinal opioid receptors. N. Engl. J. Med. 345, 935–940.

Tavani, A., Bianchi, G., Ferretti, P., Manara, L., 1980. Morphine is most effective on gastrointestinal propulsion in rats by intraperitoneal route: evidence for local action. Life Sci. 27, 2211–2217.

Wolff, B.G., Michelassi, F., Gerkin, T.M., Techner, L., Gabriel, K., Du, W., Wallin, B.A., 2004. Alvimopan, a novel, peripherally acting μ opioid antagonist. Ann. Surg. 240, 728–735.

Yuan, C.-S., Foss, J.F., O'Connor, M., Toledano, A., Roizen, M.F., Moss, J., 1996. Methylnaltrexone prevents morphine-induced delay in oral–cecal transit time without affecting analgesia: a double-blind randomized placebo-controlled trial. Clin. Pharmacol. Ther. 59, 469–475.

Yuan, C.-S., Foss, J.F., O'Connor, M., Karrison, T., Osinski, J., Roizen, M.F., Moss, J., 2000. Effects of enteric-coated methylnaltrexone in preventing opioid-induced delay in oral–cecal transit time. Clin. Pharmacol. Ther. 67, 398–404.

Zimmerman, D.M., Gidda, J.S., Cantrell, B.E., Schoepp, D.D., Johnson, B. G., Leander, J.D., 1994a. Discovery of a potent, peripherally selective *trans*-3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid antagonist for the treatment of gastrointestinal motility disorders. J. Med. Chem. 37, 2262–2265.

Zimmerman, D.M., Gidda, J.S., Cantrell, B.E., Werner, J.A., Parli, C.J., Franklin, R.B., Francis, P.C., Leander, J.D., 1994b. LY246736 dihydrate: μ opioid receptor antagonist. Drugs Future 19, 1078–1083.