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Interaction of Bivalent Ligand KDN21 with Heterodimeric δ-κ Opioid Receptors in HEK293 Cells

Zhihua Xie, Rashmi G. Bhushan, David J. Daniels, and Philip S. Portoghese

Department of Medicinal Chemistry, College of Pharmacy,

University of Minnesota, Minneapolis, Minnesota

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Interaction of KDN21 with δ and κ heterodimers

Philip S. Portoghese 308 Harvard St SE, Minneapolis, MN 55455, 612-6249174 (office): 612-6266891 (fax), porto001@maroon.tc.umn.edu

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Abbreviations:

GPCR, G protein-coupled receptors; CDK, coexpressed δ-κ opioid receptors; MDK, mixed δ and κ opioid receptors which are singly expressed; KDN#, δ-κ opioid antagonist bivalent ligand, K = κ pharmacophore, D = δ pharmacophore, N = antagonist, # refers to the number of atoms in the spacer; BNTX, 7-benzylidenenaltrexone, a δ_1 antagonist; norBNI, norbinaltorphimine, a non selective κ antagonist; NTI, naltrindole, a non selective δ antagonist; ERK1/2, extrcellular signal regulated protein kinases 1 and 2; MAPKs, mitogen-activated protein kinases.

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Abstract

KDN21 is a bivalent ligand that contains δ and κ opioid antagonist pharmacophores linked through a 21-atom spacer. It has been reported that KDN21 bridges δ and κ receptors that are organized as heterodimers. Previously, we showed that, when using [^{3}H]diprenorphine as radioligand, KDN21 displayed greatly enhanced affinity in this series for coexpressed δ and κ opioid receptors (CDK). The present study employed in vitro expression systems to investigate interactions of members of the KDN series with δ-κ heterodimers through competition binding using selective ligands and the MAPK assay. In this regard, the use of the selective radioligands, [^{3}H]naltrindole and [^{3}H]norBNI, in competition binding studies revealed that KDN21 has much higher affinity than other KDN members for CDK and bound to CDK more selectively relative to mixed δ and κ opioid receptors (MDK) or singly expressed δ (DOR) and κ opioid receptors (KOR). Other experiments revealed that the binding of naltrindole to δ opioid receptors could increase the binding of norBNI to κ opioid receptors, and vice versa, suggesting reciprocal allosteric modulation of receptors in the heterodimer. Regarding the selectivity of KDN21 for phenotypic δ and κ opioid receptors, we have investigated the effect of KDN21 upon the activation of MAPKs (ERK1/2) by δ or κ selective agonists. KDN21 inhibited the activation of ERK1/2 by DPDPE (δ_1) and bremazocine (κ_2) but had no effect on the activation by Deltorphin II (δ_2) and U69593 (κ_1). BNTX (δ_1) and bremazocine, significantly reduced the binding of KDN21 to CDK whereas NTB (δ_2) and U69593 (κ_1) produced no such change. Taken together, the data support the idea that the organization of δ and κ receptors as heterodimers gives rise to δ_1 and κ_2 phenotypes.

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Introduction

G-protein-coupled receptors (GPCRs) are classically considered to function as monomers. However, such a model has been challenged by a growing number of studies that have implicated the existence of GPCR dimers/oligomers in cultured cells and in the native state. (McLachie et al., 1998; Zawarynski et al., 1998; Zeng and Wess, 1999; George et al., 2000; for reviews, see Salahpour et al., 2000; Gomes et al., 2001; Dean et al., 2001). Opioid receptors belong to rhodopsin receptor family of GPCRs. All three major types (δ, κ, μ) of opioid receptors share high homology (~60%) and a highly conserved trans-membrane domain (for review, see Knapp et al., 1995; Dhawan BN et al., 1996; Waldhoer M et al., 2004). Prior to the development of opioid receptor cDNAs, a number of investigations suggested that opioid receptors physically interact with one another (Erez et al., 1982; Rothman and Westfall, 1982; Portoghese et al., 1986; Porreca et al., 1992; for a review, see Traynor and Elliott, 1993). With the availability of cDNAs of a great number of studies using in vitro expression systems have afforded more convincing evidence for dimerization/oligomerization of opioid receptors (Ramsay et al., 2002; Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2000). In addition, several recent studies have suggested that heterodimerization of δ and κ opioid receptors may occur in the spinal cord (Garzon et al., 1995; Wessendorf et al., 2001; Portoghese and Lunzer, 2003). However, it is still not clear how such dimeric opioid receptors are organized and what role they play in signaling. An important step toward this goal would be the development of pharmacological tools that selectively target opioid receptor heterodimers. We have very recently reported on the design and synthesis of a bivalent ligand (KDN21) which may be useful in this regard (Bhushan et al., 2004). KDN21 contains δ and κ opioid receptor antagonist pharmacophores that are connected through a 21-atom spacer (Fig.

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1). Intrathecal pharmacological studies in mice and binding studies in HEK293 cells have suggested that KDN21 bridges δ and κ opioid receptors associated as heterodimers (Bhushan et al., 2004). The results of studies with selective agonists and antagonists in vivo are consistent with cooperativity between δ and κ opioid receptors, and it has been proposed that the putative δ_1 and κ_2 opioid receptor subtypes are actually phenotypes (Portoghese et al., 2003). We previously reported that the affinity of KDN21 for δ and κ opioid receptors coexpressed in HEK293 cells (CDK) was substantially greater relative to other members of the KDN series containing longer or shorter spacers, when [^{3}H]diprenorphine was employed as radioligand (Bhushan et al., 2004). Although the in vitro binding data was consistent with the in vivo study, it did not identify the binding characteristics of each of the bridged receptors. Using [^{3}H]naltrindole and [^{3}H]norBNI as radioligands in the present study, we have identified the receptors bound by KDN21 and confirmed that the 21-atom spacer in the KDN series confers optimal bridging to dimerized δ and κ opioid receptors. Furthermore, we have obtained evidence for cooperativity between the receptors in the heterodimer through the facilitated binding of [^{3}H]naltrindole or [^{3}H]norBNI in the presence of norBNI or naltrindole, respectively. Finally, we have demonstrated that KDN21 is selective in its ability to antagonize opioid agonist-induced activation of the mitogen-activated protein kinase (MAPK) signaling cascade. Significantly, KDN21 antagonized only the δ_1 and κ_2 selective agonists that activated MAPKs (ERK1/2), in conformity with its pharmacological selectivity in vivo. The present study indicates that KDN21 may become a useful tool in opioid research.

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Materials and methods

Materials. Cell culture and transfection reagents, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, G418 (geneticin), hygromycin, streptomycin, penicillin, Trypsin/EDTA and Lipofectin Reagent were purchased from Invitrogen Corporation (Carlsbad, CA). [^{3}H]diprenorphine (50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). [^{3}H]naltrindole (35 Ci/mmol), [^{3}H]norBNI (9.08 Ci/mmol) were supplied by the National Institute on Drug Abuse (NIDA) of National Institute of Health (Bethesda, MD). U69593, naloxone and Protease Inhibitor Cocktail was purchased from Sigma Aldrich (St. Louis, MO). 40% Acrylamide Stock (29:1), ammonium persulfate and TEMED were obtained from Fisher Scientific (Hampton, NH). Econo-Safe scintillation cocktail was from Research Products International (RPI) Corp. (Mount Prospect, IL). Modified Lowry Protein Assay Reagent Kit was purchased from Pierce Biotechnology (Rockford, IL). Anti-Active MAPK pAb and Donkey Anti-Rabbit IgG were purchased from Promega Corporation (Madison WI). Full Range Rainbow^{TM} marker and ECF substrate for Western blotting were purchased from Amersham Biosciences (Piscataway, NJ). BNTX, NTB, bivalent ligands KDN15, 20, 21, 22, 23, 24, and monovalent controls DN21 and KN21 were synthesized in our laboratory. Plasmid cDNAs of δ and κ opioid receptors were kindly provided by Dr. Lakshmi Devi and Carl Rios of New York University. Bremazocine and stable DOR HEK293 cell line was gifted by Dr. Ping Law of University of Minnesota. Stable KOR HEK293 cell line was kindly provided by Lee-Yuan Liu-Chen of Temple University.

Cell culture and stable expression of the receptors in HEK293 cells. cDNAs encoding rat δ and mouse κ opioid receptors were inserted separately into the mammalian expression vector pcDNA3. The receptors were tagged with different epitopes respectively: a 10-residue c-Myc

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epitope (EQKLISEEDL) for the δ opioid receptors and an 8-residue Flag epitope (DYKDDDDK) for the κ opioid receptors. Human embryonic kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 unit/ml penicillin under humidified atmosphere in a 10% CO_2 incubator at 37°C. The cells, grown to about 50% confluence, were transfected with the expression vectors containing δ or κ opioid receptor cDNA. Transfections were done in serum-free medium using Lipofectin Transfection Kit according to the supplier's mannual. We used 10 μg of cDNA to transfect the cells of each 100 mm plate. For coexpression, the cells were first transfected with the pcDNA3 vector of δ opioid receptor and then with that of κ opioid receptor. The cells were incubated in transfection medium for 24 h and then changed to be incubated in complete DMEM medium for another 24 h. The cells were split and transfered into selective medium for culture until the appearance of stable cell colony. The expression level of the receptors was represented by their maximum binding to indicated radioligand (B*max*). The B*max* values of the singly expressed DOR and KOR in this study were 1091.5 and 1212.6 fmol/mg protein separately, and the DOR and KOR cells were mixed at 1:1 ratio of B*max* for control MDK. The cells coexpressing δ and κ opioid receptors close to 1:1 (*Bmax* of δ and κ opioid receptors are 854.2 and 879.3 fmol/mg protein separately) were chosen for experiment purposes.

Determination of protein concentration and receptor ratio. The modified Lowry protein assay was employed to determine protein concentration of our samples, using bovine serum albumin (BSA) for establishing a standard curve of absorbance versus micrograms of protein. The sample was determined in triplicate and the average absorbance value was used to determine the protein concentration of the same sample through comparison with the standard.

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We employed 5 nM of [^{3}H]diprenorphine and [^{3}H]naltrindole as tools to determine the ratio of δ and κ opioid receptors. Saturation binding assay was employed to determine the maximal binding of [^{3}H]diprenorphine and [^{3}H]naltrindole (*B*max) to the cells coexpressing δ and κ opioid receptors. The *B*max was expressed as fmol/mg of protein. The *B*max of [^{3}H]diprenorphine was the total binding to δ and κ opioid receptors, and *B*max of [^{3}H]naltrindole represented the binding to δ opioid receptors. The difference between the *B*max of [^{3}H]diprenorphine and the *B*max of [^{3}H]naltrindole represented the binding to κ opioid receptors.

Radioligand Binding Assay. Cells were grown to about 90% confluence in 100 mm diameter dishes, collected using ice-cold Trypsin/EDTA, and centrifuged at 1,000*g* for 5 min. The cell pellets were rinsed once with ice-cold PBS buffer (pH 7.4) and centrifuged at 1,000*g* for 5 min again. The cells were re-suspended in binding buffer (25 mM HEPES, 120mM NaCl, 1.5mM CaCl2, 5mM KCl, 1.5mM MgCl2, pH7.4). Saturation binding was conducted on intact whole cells using radioligands to determine receptor density (*Bmax*) and binding affinity (*Kd*) of the ligand. The cells were incubated with increasing concentrations of a radioligand at 25°C for 2 h in a total volume of 500 μl reaction system. Each concentration was performed in duplicate and nonspecific binding was defined as the binding of the radioligand not displaced by 10 μM of naloxone. The IC_{50} values for tested compounds were determined by competition binding assay in which whole cells were incubated at 25°C for 2 h with an indicated radioligand and 9 different concentrations (10^{-14}-10^{-6} M) of the compounds in a final volume of 500 μl. The concentration of the radioligand employed in the competition assay was approximately equivalent to its *Kd,* and nonspecific binding was determined as in the presence of 10 μM of naloxone. The experiments were repeated three times in duplicate for each assay.

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Bound radioligand was separated from the free by rapid filtration through Brandel 48-well harvester using Whatman GF/C glass fiber filter paper, presoaked in 0.25% solution of PEI in water. The filters with trapped cells were rinsed three times with 4.0 ml of 25 mM HEPES buffer (pH 7.4) precooled to 4^{o}C and placed in scintillation vials with 4.0 ml of Econo-Safe scintillation cocktail and counted in LS3801 Beckman counter for 1 min/sample.

MAP kinase activity assay. Cells were grown to about 90% confluence in 100 mm diameter dishes, serum-free starved for 6 h, and then incubated with the compounds at 25°C for 10 min. The use of serum-free medium has been shown to reduce the basal level of MAPK activation by inhibiting enzyme phosphorylation (Polakiewicz et al., 1998). Cells were collected using ice-cold Trypsin/EDTA and centrifuged at 1,000*g* for 5 min. The cell pellets were rinsed once with ice-cold PBS and centrifuged at 1,000*g* for 5 min again. Cell lysates were prepared by addition of appropriate volume of ice-cold cell lysis buffer (50 mM Tris, 300 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4) containing 1× Protease Inhibitor Cocktail. The lysates were centrifuged in a Microfuge at 12,000*g* for 10 min at 4°C. The supernatants were analyzed for protein concentration using the Modified Lowry Protein Assay and then were prepared for SDS–polyacrylamide gel electrophoresis (PAGE) by boiling them in the sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.05% bromophenol blue, pH 6.8). Proteins (~10 mg/lane) were separated by SDS–PAGE (10% acrylamide running gel, 4% acrylamide stacking gel) and electroblotted onto PVDF membrane (0.45 μm). The PVDF membrane was rinsed 3 times with TTBS buffer (25 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) and blocked overnight (~12 h) at 4°C in TTBS of 5% non-fat dry milk. Then the PVDF membrane was incubated at 25°C for 2 h with rabbit polyclonal antibodies against phosphorylated MAPK

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(1:5,000 dilution in blocking solution), and after washed 5 times, 5 min each time, incubated at 25°C for 1 h with donkey anti-rabbit secondary antibodies conjugated to alkaline phosphatase (1:5,000). Immuno-reacted proteins were visualized using an alkaline phosphatase sensitive ECF substrate at 25°C for 10 min.

Data analysis and statistical methods. Saturation and competition radioligand binding curves were generated through Kaleidgraph 3.1. The *Bmax* and *Kd* values of the radioligand were determined by Scatchard Plotting analysis, where the X axis is specific binding and the Y axis is specific binding divided by free radioligand concentration. IC_{50} values were determined from displacement curves using Kaleidgraph 3.1, and the *Ki* values were calculated according to the Cheng–Prussoff equation Ki = IC50/1+[L]/Kd (Cheng and Prusoff, 1973). Images of immunoreactive bands were captured on Storm system from Molecular Dynamics, and the activity of the MAPKs was represented by the gray values of the protein bands on the PVDF membrane. The software of NIH image 1.61 was used to quantify the phosphorylated MAPKs. Statistical data is expressed as mean $\pm$ S.E. of the indicated number of observations. Student's t-test was employed to compare the difference between the samples for significance tests.

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Results

Binding of KDN bivalent ligands to coexpressed δ and κ opioid receptors in HEK293 cells. In a prior report we had evaluated the KDN series of bivalent ligands using in vitro expression systems. We found that KDN21 possessed the greatest affinity for coexpressed δ and κ opioid receptors when [^{3}H]diprenorphine was employed as radioligand, (Bhushan et al., 2004). However, since [^{3}H]diprenorphine is a non-selective ligand, we were not able to obtain the information on the binding of KDN21 to individual receptors in the heterodimer. In this study, we have employed δ selective antagonist, [^{3}H]natrindole (0.1 nM), and κ selective antagonist, [^{3}H]norBNI (2.0 nM), as radioligands to further evaluate the binding of KDN series members to CDK, MDK, DOR and KOR in HEK293 cells. It is noteworthy that the binding of [^{3}H]naltrindole and [^{3}H]norBNI to coexpressed and singly expressed receptors was similar. The greatly reduced affinity reported (Jordan and Devi, 1999) for the binding of selective ligands to δ–κ heterodimers may have been due to the use of the non-selective radioligand, [^{3}H]diprenorphine. As shown in Table 1, when using [^{3}H]natrindole as label, KDN21 had greater binding affinity for CDK than other KDN members, and relative to MDK and DOR, their *Ki* values differing by more than one order of magnitude. KDN21 also bound more avidly to CDK than other members and relative to MDK and KOR when [^{3}H]norBNI was employed as label. Other bivalent ligands in this series had similar binding affinity for CDK, MDK, DOR, or KOR. The monovalent δ antagonist DN21 and monovalent κ antagonist KN21 exhibited poor ability to displace the binding of [^{3}H]norBNI and [^{3}H]natrindole, respectively.

Binding of naltrindole and norBNI to coexpressed δ and κ opioid receptors in HEK293 cells. Although there has been growing evidence supporting the dimerization/oligomerization of coexpressed opioid receptors, little is known about the roles that

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dimerization/oligomerization play in the regulation of opioid receptors. We have employed the δ selective antagonist [^{3}H]naltrindole and κ selective antagonist [^{3}H]norBNI to evaluate allosteric effect on the binding of these radioligands to HEK293 cells containing coexpressed δ and κ opioid receptors. We first evaluated their direct binding to CDK. Both [^{3}H]naltrindole and [^{3}H]norBNI showed good affinity for that (Fig. 2A and 2B). Their *Kd* values were 0.1 ± 0.04 nM and 2.0 ± 0.88 nM respectively, which were very close to their *Kd* values for singly expressed δ or κ opioid receptors DOR or KOR (*Kd* of [^{3}H]naltrindole for DOR was 0.13 ± 0.05 nM and *Kd* of [^{3}H]norBNI for KOR was 1.56 ± 0.72 nM). This suggested that the dimerized δ and κ opioid receptors retained binding to their own selective ligands. In order to evaluate possible allosteric interaction between δ and κ opioid receptors, we have further investigated the effects of cold norBNI (10 nM) on the binding of [^{3}H]naltrindole and cold naltrindole (10 nM) on the binding of [^{3}H]norBNI to the coexpressed and mixed δ and κ opioid receptors (CDK and MDK) in HEK293 cells. In CDK cells, naltrindole was found to increase the binding affinity of [^{3}H]norBNI by 36-fold (Fig. 2A), similarly, norBNI increased the binding affinity of [^{3}H]naltrindole by 27-fold (Fig. 2B). Significantly, in MDK cells, no such facilitation change occurred (Fig. 2C and 2D).

Effects of δ and κ selective ligands on the binding of KDN21 to coexpressed δ and κ opioid receptors in HEK293 cells. Based on the above binding data and our previous studies (Bhushan et al., 2004), in the KDN series, KDN21 is an optimal candidate to bridge δ and κ opioid receptors in vivo and in vitro. In CDK cells, we have found that δ_1, δ_2, κ_1 and κ_2 pharmacological phenotypes coexist, based on the activation of ERK1/2 of MAP kinases by different selective agonists (Table 2). In order to determine the selectivity of KDN 21 for such phenotypic receptors, we employed selective δ and κ opioid ligands as tools to evaluate their

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effects on the binding of KDN21 to CDK. When using 0.1 nM of [^{3}H]naltrindole as radioligand, in the presence of κ_2 agonist, bremazocine (100 nM), the binding curve of KDN21 to CDK was shifted to the right (Fig. 3A), and its *Ki* value increased 25-fold compared to that in the absence of bremazocine, whereas κ_1 agonist, U69593 (100 nM) had little influence. When using 2 nM of [^{3}H]norBNI in the presence of δ_1 antagonist, BNTX (100 nM), the binding curve of KDN21 to CDK was also shifted right (Fig. 3B). Its *Ki* value was 18-fold higher than that in the absence of BNTX. Significantly, the δ_2 antagonist, NTB (100 nM), had little influence in this case. In the both cases, the binding of KDN21 to MDK were not changed by δ or κ selective ligand (Fig 3C and Fig. 3D). Since BNTX and bremazocine inhibited the binding of KDN21 to CDK cells, but NTB and U69593 did not, this suggests that the bridged receptors are δ_1 and κ_2 phenotypic receptors and provides additional support to our reported (Bhushan et al., 2004) intrathecal studies that revealed that KDN21 selectively antagonized the antinociception induced by administration of DPDPE and bremazocine.

Effect of KDN 21 on phosphorylation of MAPKs activated by δ or κ selective agonists. Many GPCRs, including all three opioid receptors μ, δ and κ, have been reported to activate the MAPKs signaling cascade (Fukuda et al., 1996; Li and Chang, 1996). MAPK activation by GPCRs allows for plasma membrane receptor systems to influence diverse cellular processes, ranging from the regulation of neuronal survival to cell differentiation and gene expression (Gutkind, 1998). Our binding data and intrathecal studies have indicated that KDN21 selectively targets the δ_1 and κ_2 phenotypic opioid receptors. In order to obtain direct evidence that KDN21 regulates the function of δ_1 and κ_2 opioid phenotypes, we employed different δ and κ selective agonists to activate the phosphorylation of ERK1/2 and then observed the antagonism of KDN21 upon such activation. In CDK cells, DPDPE, deltorphin II, U69593 and

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bremazocine could stimulate the phosphorylation of ERK1/2 (Table 2). Compared to the control, they could raise the phosphorylation level of ERK1/2 2-3 fold. 100 nM of KDN21 could inhibit the activation of ERK1/2 induced by 100 nM of DPDPE or 100 nM of bremazocine, and the magnitude of inhibition was about 2.3 fold ($p<0.01$). However, no significant inhibition on the activation induced by 100 nM of deltorphin II and 100 nM of U69593 was observed. These results give direct evidence for the regulation of KDN21 on the function of δ_1 and κ_2 opioid phenotypes.

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Discussion and Conclusion

An important issue in opioid research is concerned with the greater number of putative receptor subtypes than revealed through cloning. Several reports implicating the existence of δ-κ heterodimeric opioid receptors in vitro and in vivo and have suggested that the putative δ1 and κ2 opioid receptor subtypes are in fact phenotypes (Portoghese and Lunzer, 2003; Bhushan et al., 2004). δ-κ opioid receptor heterodimers appeared to display molecular recognition properties that differ from those of their homomeric counterparts (Jordan and Devi, 1999; Bhushan et al., 2004; Daniels et al., 2004). Most recently, this difference has raised the possibility of developing ligands that activate δ-κ heterodimers.

In the present study, we have employed a bivalent ligand, KDN21, that contains δ and κ antagonist pharmacophores linked through a 21-atom spacer, as a tool to investigate the properties of heterodimers of δ and κ opioid receptors in HEK293 cells. In this regard, KDN21 has been reported to have a spacer of optimal distance for bridging δ and κ opioid receptors organized as heterodimers (Bhushan et al., 2004). Although KDN21 possessed the highest affinity for coexpressed δ and κ opioid receptors in HEK293 cells and its in vivo pharmacological selectivity corresponded to the δ_1 and κ_2 phenotypes, the phenotypic receptors were not identified in binding studies because nonselective [^{3}H]diprenorphine was used as a radioligand. Consequently, in this study we have employed selective radioligands to investigate the binding selectivity. With the δ antagonist, [^{3}H]naltrindole, or κ antagonist, [^{3}H]norBNI, coexpressed δ and κ opioid receptors retained good binding selectivity for these selective ligands (Table 1). Using either radioligand, KDN21 showed much higher binding affinity than other members of the series for HEK293 cells coexpressed with both δ and κ receptors (CDK).

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This provides more direct evidence for the optimal bridging of δ and κ opioid receptors by KDN21.

Pharmacological studies have suggested cooperative interaction between δ and κ opioid receptors, and allosteric coupling has been proposed based on the finding that the κ-selective antagonist, norBNI antagonizes the δ_1 agonist, DPDPE, in the spinal cord (Portoghese and Lunzer, 2003). A similar type of antagonism has been reported in the porcine ileum preparation (Poonyachoti et al., 2001). In the both cases, the presence of δ and κ receptors in the same cell has provided support for antagonism being mediated via allosteric heterodimers rather than through cellular circuitry. In the present study, the enhanced affinity of [^{3}H]naltrindole in the presence of norBNI suggests that the δ and κ opioid receptors organized as heterodimers function cooperatively (Fig. 2A). That this was a reciprocal effect was demonstrated by the increased affinity of [^{3}H]norBNI in the presence of naltrindole (Fig. 2B). These results provide more direct evidence that δ and k opioid receptors function cooperatively as heterodimers. According to the conceptual model illustrated in figure 4, δ-κ opioid receptor heterodimers exist either in the agonist or antagonist state, but not the mixed state. Interaction of a selective antagonist will therefore transform both δ and κ receptor subunits to the antagonist state. Thus, a δ antagonist can antagonize a kappa agonist and a κ antagonist can antagonize a δ agonist, both allosterically. The allosteric nature of such coupling has far-reaching implications because antagonism of an agonist by a selective antagonist does not necessarily reveal the receptor that mediates the agonist effect. Given the above results, it seems likely that δ and κ opioid receptor subunits in the heterodimer also facilitate binding of KDN21 when the spacer permits optimal bridging of the pharmacophores. The binding data for the KDN series (Table 1) are consistent with this view if it is assumed that bridging of KDN21 to the opioid recognition sites on the

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heterodimer occurs in discrete steps involving univalent binding followed by the binding of the second pharmacophore (Fig. 5). KDN21 most likely possesses greatly increased affinity relative to other bivalent and monovalent ligands in the series through a combination of positive allosterism and high local concentration of one of the free pharmacophores in the vicinity of the vacant neighboring receptor in the heterodimer. The lower affinity of KDN21, in the presence of κ_2 selective agonist bremazocine or δ_1 selective antagonist BNTX (Fig. 3), is possibly attributable to the competition of them with KDN21 for one of the two binding sites in the heterodimer. Such competition could change the binding of KDN21 from a bivalent state to a univalent state and therefore lead to a lower affinity of KDN21.

Intrathecal studies in mice have revealed that KDN21 displays δ_1 and κ_2 selectivity. This was based on the antagonism of the δ_1 agonist, DPDPE and the κ_2 agonist, bremazocine. In this regard, the δ_2 agonist, deltorphin II, and the κ_1 agonist, U69593, were only weakly antagonized by KDN21. When taken together with the reports that norBNI antagonizes both DPDPE and bremazocine, it was concluded that KDN21 interacts selectively with allosteric heterodimeric δ-κ opioid receptors whose phenotypes correspond to δ_1 and κ_2, respectively. In order to determine whether or not KDN21 effects on the same phenotypic opioid receptors in CDK HEK293 cells, we investigated the antagonism of selective agonist-stimulated phosphorylation of MAPKs (ERK1/2) by KDN21. Significantly, similar antagonist selectivity was observed, in that KDN21 antagonized δ_1 and κ_2 selective agonists in both HEK293 cells and in mice upon intrathecal administration. No significant antagonism of the δ_2 and κ_1 agonists was observed. These results have provided support for the existence of δ_1 and κ_2 phenotypic opioid receptors in HEK293 cells. Moreover, the qualitatively similar antagonist selectivity of KDN21 in CDK-

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HEK293 cells lends additional support to the study that KDN21 selectively interacts with δ-κ opioid receptor heterodimers in the mouse spinal cord.

In conclusion, our studies with the bivalent ligand, KDN21 provides new insights into the interaction of ligands with δ-κ opioid receptor heterodimers that recognize δ_1- and κ_2-selective ligands. These phenotypic receptors are allosterically coupled and recognize agonists such as DPDPE and bremazocine. The cooperative nature of these heterodimers is manifested by the reported antagonism of a δ_1 agonist (DPDPE) by a κ antagonist (norBNI) or the antagonism of a κ_2 agonist (bremazocine) by a δ_1 antagonist (BNTX). In view of these findings, the assignment of a receptor type based upon the selectivity of a standard selective opioid antagonist can be problematic. Finally, KDN21 should be a useful tool for identifying δ-κ opioid receptor heterodimers both in vitro and in vivo.

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References

Bhushan RG, Sharma SK, Xie Z, Daniels DJ, Portoghese PS (2004) A bivalent ligand (KDN-21) reveals spinal δ and κ opioid receptors are organized as heterodimers that give rise to δ1 and κ2 phenotypes. Selective targeting of δ-κ heterodimers. J Med Chem 47:2969-72.

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzyme reaction. Biochem Pharrmacol 22:3099-3102.

Daniels DJ, Kulkarni A, Xie Z, Bhushan RG, and Portoghese PS (2005) A Bivalent Ligand (KDAN-18) Containing δ Antagonist and κ Agonist pharmacophores Bridges δ2 and κ1 Opioid Receptor Phenotypes. J Med Chem 48:1713-1716.

Dean MK, Higgs C, Smith RE, Bywater RP, Snell CR, Scott PD, Upton GJ, Howe TJ and Reynolds CA (2001) Dimerization of G-protein coupled receptors. J Med Chem 44:4595-4614.

Dhawan BN, Cesselin F, Raghubir R, Reisine T, Bradley PB, Portoghese PS, Hamon M (1996) International Union of Pharmacology. XII. Classification of opioid receptors. Pharmacol Rev 48:567-92.

Erez M, Takemori AE, Portoghese PS (1982) Narcotic antagonistic potency of bivalent ligands which contain β-naltrexamine. Evidence for bridging between proximal recognition sites. J Med Chem 25:847-849.

Fukuda K, Kato S, Morikawa H, Shoda T, Mori K (1996) Functional coupling of the δ, μ-, and κ opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. J Neurochem 67:1309-1316.

Garzon J, Juarros JL, Castro MA, Sanchez-Blazquez P (1995) Antibodies to the cloned μ-

opioid receptor detect various molecular weight forms in areas of mouse brain. Mol Pharmacol 47:738-744.

George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF (2000) Oligomerization of μ and δ opioid receptors:gneration of novel functional properties. J Biol Chem 275:26128-226135.

Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA (2000) Heterodimerization of μ and δ opioid receptors: a role in opiate synergy. J Neurosci 20: RC110.

Gutkind JS (1998) The pathway connecting G-protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J Bio Chem 273:1839-842.

Jordan BA, Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399: 697-700.

Knapp RJ, Malatynska E, Collins N, Fang L, Wang JY, Hruby VJ, Roeske WR and Yamamura HI (1995) Molecular biology and pharmacology of cloned opioid receptors. FASEB J 9:516-525.

Li LY, Chang KJ (1996) The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express μ opioid receptors. Mol Pharmacol 50:599-602.

McLachie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Ford SM (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393:333-339.

Polakiewicz RD, Schieferl SM, Dorner LF, Kansra V, Comb MJ (1998) A mitogen-activated protein kinase pathway is required for μ opioid receptor desensitization. J Bio Chem 273:12402-12406.

Poonyachoti S, Portoghese PS, Brown DR (2001) Characterization of opioid receptors modulating neurogenic contractions of circular muscle from porcine ileum and evidence that delta- and kappa-opioid receptors are coexpressed in myenteric neurons. J Pharmacol Exp Ther 297:69-77.

Porreca F, Takemori AE, Sultana M, Portoghese PS, Bowen WD, Mosberg HI (1992) Modulation of mu-mediated antinociception in the mouse involves opioid delta-2 receptors. J. Pharmacol Exp Ther 263:147-152.

Portoghese PS, Larson DL, Sayre LM, Yim CB, Ronsisvalle G, Tam SW, Takemori AE (1986) Opioid agonist and antagonist bivalent ligands. The relationship between spacer length and selectivity at multiple opioid receptors. J Med Chem 29:1855-1861.

Portoghese PS, Lunzer MM (2003) Identity of the putative δ1 opoiod receptor as a β-κ heteromer in the mouse spinal cord. Euro J Pharmacol 467:233-234.

Ramsay D, Kellett E, McVey M, Rees S, Milligan G (2002) Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. Biochem J 365:429-40.

Rothman RB, Westfall TC (1982) Allosteric coupling between morphine and enkephalin receptors in vitro. Mol Pharmacol. 21:548-57.

Salahpour A, Angers S, Bouvier M (2000) Functional significance of oligomerization of G protein-coupled receptors. Trends Endocrin Met 11:163-168.

Traynor JR, Elliott J (1993) δ opioid receptor subtypes and cross-talk with μ receptors. Trends Pharmacol Sci 14:84-86.

Waldhoer M, Bartlett SE, Whistler JL (2004) Opioid receptors. Annu Rev Biochem 73:953-90

Wessendorf MW, Dooyema J (2001) Coexistence of κ and δ opioid receptors in rat spinal cord axons. Neurosci Lett 298:151-154.

Zeng FY, Wess J (1999) Identification and molecular characterization of m3-muscarinic receptor dimmers. J Biol Chem 274:19487-19497.

Zawarynski P, Tallerico T, Seeman P, Lee SP, O'Dowd BF, George SR (1998) Dopamine D2 receptor dimmers in human and rat brain. FEBS Lett 441:383-386.

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Fig. 1 Stucture of the Bivalent ligand KDN21. KDN21 is composed of a δ antagonist pharmacophore, NTI (Bhushan et al., 2004), and a κ antagonist pharrmacophore, GNTI (Bhushan et al., 2004), linked through a spacer of 21 atoms. The spacer is comprised of oligoglycyl units attached to a succinyl core.

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Fig. 2 Binding of naltrindole and norBNI to δ and κ opioid receptors coexpressed in HEK293 cells. The binding assay was conducted on intact whole cells using [^{3}H]norBNI and [^{3}H]naltrindole as radioligand. Each assay was performed in duplicate and the data shown here are representative of three independent experiments (means ± S.E.). The employed concentration of cold naltrindole and cold norBNI was 10 nM. The cold naltrindole facilitated the binding of [^{3}H]norBNI to κ opioid receptors in CDK (A) - The binding affinity of [^{3}H]norBNI in the presence of naltrindole (-●-) increases significantly relative to its affinity in the absence of naltrindole (-○-). Similarly, the cold norBNI facilitates the binding of [^{3}H] naltrindole to δ opioid receptors in CDK (B) - The binding affinity of [^{3}H]naltrindole in the presence of norBNI ((-●-) increases significantly relative to its affinity in the absence of norBNI (-○-). Significantly, in MDK cells, no such facilitation change occurred (Fig. 2C and 2D)

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Fig. 3 Effects of δ and κ selective ligands on the binding of KDN21 to the CDK. The binding assay was conducted on intact whole cells using [^{3}H]naltrindole or [^{3}H]norBNI as radioligand. Each assay was performed in duplicate, and the data shown here are representative of three independent experiments (means ± S.E.). The concentration of [^{3}H]naltrindole and [^{3}H]norBNI was 0.1 nM and 2.0 nM respectively, and the concentration of U69593, bremazocine, BNTX and NTB was 100nM. When using [^{3}H]naltrindole as label, bremazocine significantly decreased the binding affinity of KDN21 for CDK, but U695593 showed little influence (A); when [^{3}H]norBNI was employed as label, BNTX significantly inhibited the biding of KDN21 to CDK, but NTB showed no such inhibition (B). In the both cases, the binding of KDN21 to MDK were not changed by δ or κ selective ligand (C and D).

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Fig. 4 A conceptional model of the antagonism of a δ_1 or a κ_2 agonist by a κ_2 or a δ_1 antagonist at a δ-κ opioid receptor heterodimer. The change from an agonist state (a) to an antagonist state (b) by the binding of a δ_1 (A) or a κ_2 selective antagonist (B) is accompanied by a conformational change which reduces the affinity of the bound κ_2 or δ_1 agonist.

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Fig. 5 A conceptional model of the interaction of KDN21 with a δ-κ opioid receptor heterodimer. The δ-κ opioid receptor heterodimer (a) is bound univalently by KDN21 (b), This is accompanied by a conformational change of both receptor subunits which facilitate binding of the second pharmacophore (c). Increased binding of KDN21 to δ-κ opioid receptor heterodimer is also probably due to proximity of the unbound pharmacophore to the vacant receptor (b). This model assumes that both subunits are either in the agonist state (a) or antagonist state (b, c).

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Table 1

Binding of KDN bivalent ligands and monovalent controls to coexpressed and singly expressed δ and κ opioid receptors

Competition binding assay was conducted on intact whole cells using 0.1 nM of [^{3}H]natrindole or 2.0 nM of [^{3}H]norBNI as radioligand. Each assay was performed in duplicate, and the data shown here are representative of at least three independent experiments (means ± S.E.). CDK is coexpressed δ-κ opioid receptors in HEK293 cells, MDK is mixed δ and κ opioid receptors which singly expressed in HEK293 cells, DOR is singly expressed δ opioid receptors and KOR is singly expressed κ opioid receptors in HEK293 cells. KDN#, δ and κ antagonist bivalent ligand, K = κ pharmacophore, D = δ pharmacophore, N = antagonist, and # = atoms in the spacer. DN21 is NTI with a spacer of 21 atoms. KN21 is GNTI with a spacer of 21 atoms.

Ligand	*Ki* (nM)					
	[^{3}H]Naltrindole			[^{3}H]norBNI		
	CDK	MDK	DOR	CDK	MDK	KOR
KDN 15	3.42 ± 1.06	3.38 ± 1.44	1.92 ± 0.68	3.62 ± 1.41	2.51 ± 1.19	1.82 ± 0.62
KDN 20	1.02 ± 0.50	3.23 ± 1.37	2.45 ± 1.09	1.90 ± 0.98	2.62 ± 1.29	2.45 ± 1.19
KDN 21	0.06 ± 0.03	1.38 ± 0.57	3.37 ± 1.46	0.16 ± 0.06	4.67 ± 2.06	3.01 ± 1.42
KDN 22	2.34 ± 1.08	2.44 ± 1.21	1.23 ± 0.58	4.56 ± 2.04	2.45 ± 1.04	2.18 ± 1.17
KDN 23	1.58 ± 0.69	3.62 ± 1.18	7.40 ± 2.20	5.24 ± 2.61	2.34 ± 1.20	2.51 ± 1.36
KDN 24	2.04 ± 0.98	7.46 ± 2.57	2.06 ± 0.73	3.88 ± 1.84	2.04 ± 0.93	1.69 ± 0.73
DN21	1.91 ± 0.84	3.29 ± 1.04	1.98 ± 0.52	137.7 ± 54.6	147.6 ± 59.8	203.7 ± 9.98
KN21	197.8 ± 67.9	137.7 ± 55.8	190.1 ± 79.9	4.35 ± 1.74	2.45 ± 1.23	1.58 ± 0.83

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Table 2

Effect of KDN21 on the activation of ERK1/2 induced by δ and κ selective agonists. HEK293 Cells coexpressing δ and κ opioid receptors were grown to about 95% confluence and exposed to serum-free media for 6 h before the addition of the indicated opioid agonist, and then incubated with 100nM of DPDPE, deltorphin II, U69593 or brmazocine in the presence or absence of KDN21 (100 nM) at 37°C for 10 min. The cells were extensively washed and prepared for MAPK extraction as mentioned in Methods section. 10 μg of total protein was separated via SDS-PAGE, followed by immunoblotting using the antibody raised against phosphorylated MAPK. NIH Image 1.61 software was used to quantify phosphorylated MAPK level. The level of phosphorylated MAPK in the absence of ligand treatment is taken as control and defined as 100%. The data are means ± S.E. of three independent experiments. * Significantly different from the MAPK level in the absence of KDN21 ($p < 0.01$).

Ligand	ERK1/2 activity (% of base)
DPDPE	304.4 ± 15.4
DPDPE + KDN21	131.0 ± 5.4 *
Deltorphin II	229.0 ± 10.1
Deltorphin II + KDN21	220.2 ± 13.4
U69593	255.7 ± 14.0
U69593 + KDN21	253.7 ± 12.7
Bremazocine	278.0 ± 11.2
Bremazocine + KDN21	119.9 ± 6.9 *

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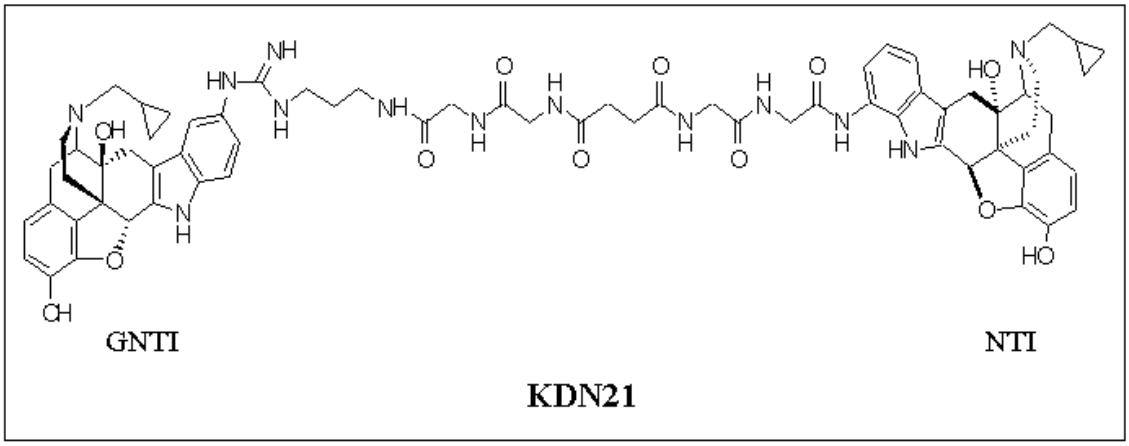

Fig. 1

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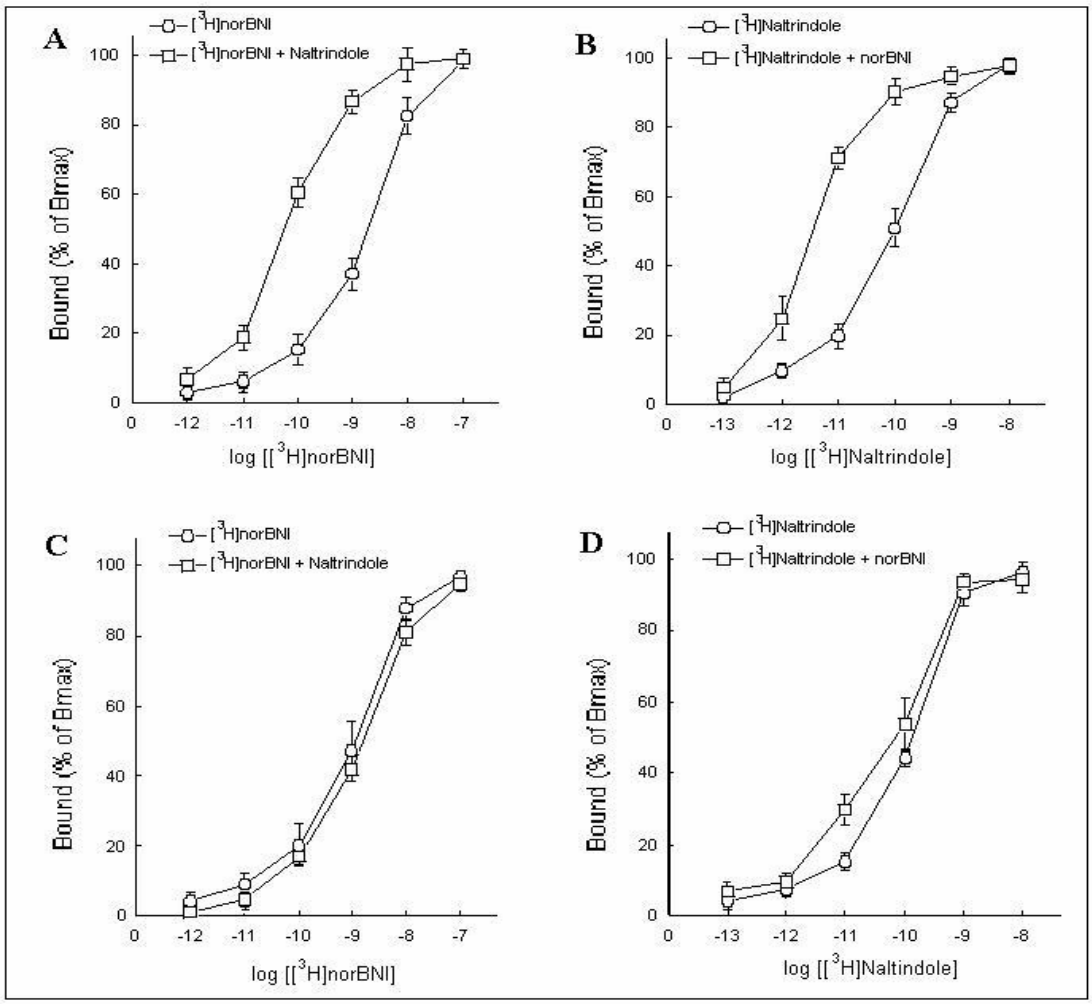

Fig. 2

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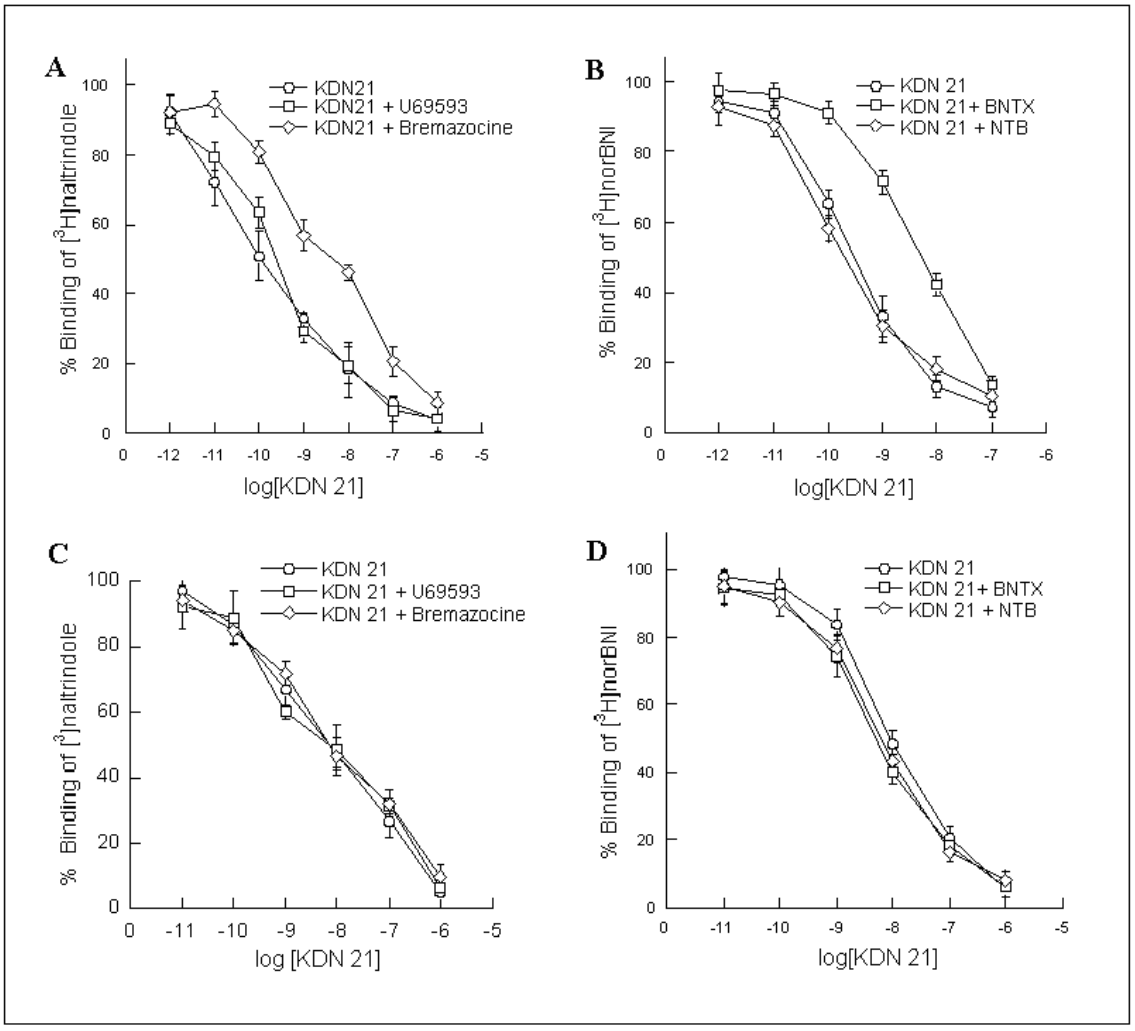

Fig. 3

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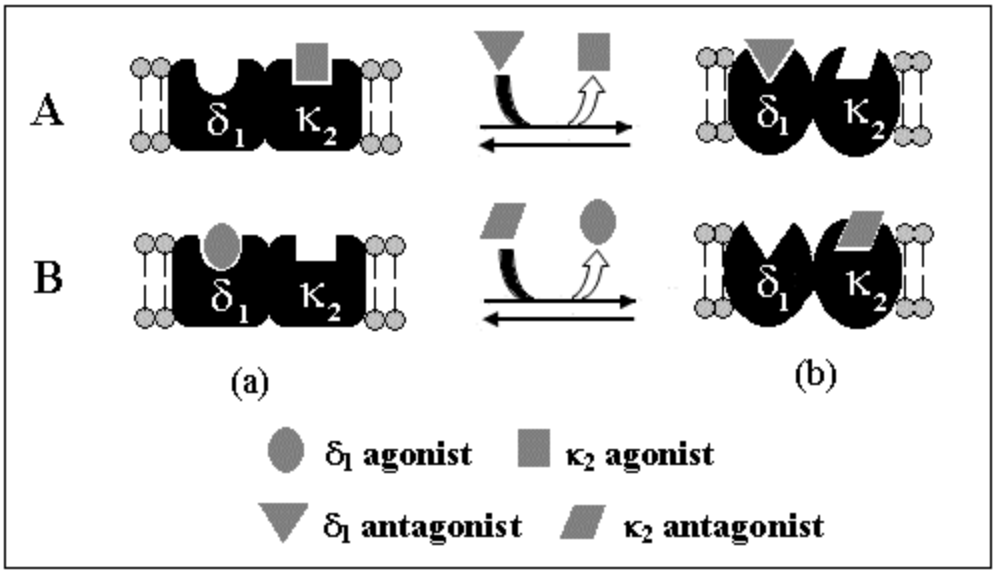

Fig. 4

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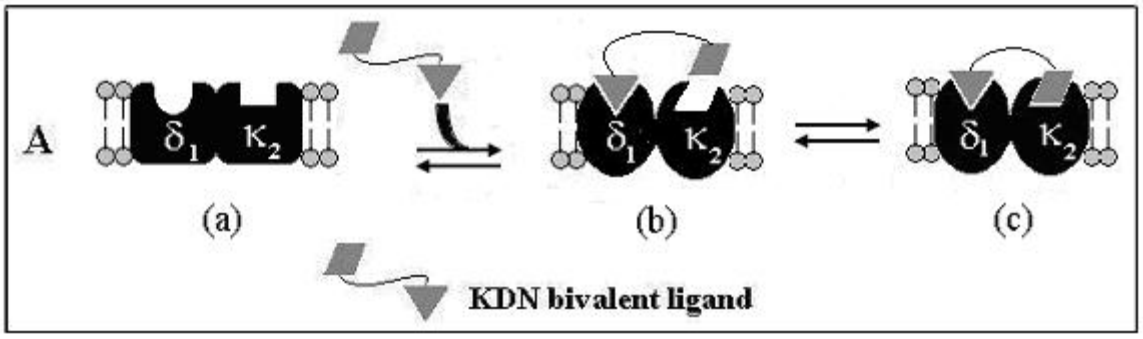

Fig.5