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# Synthesis and characterizations of novel quinoline derivatives having mixed ligand activities at the κ and μ receptors: Potential therapeutic efficacy against morphine dependence

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ABSTRACT

Based on an established 3D pharmacophore, a series of quinoline derivatives were synthesized. The opioidergic properties of these compounds were determined by a competitive binding assay using $^{125}$I-Dynorphine, $^{3}$H-DAMGO and $^{125}$I-DADLE for κ, μ, and δ receptors, respectively. Results showed varying degree of activities of the compounds to κ and μ opioid receptors with negligible interactions at the δ receptor. The compound, S4 was the most successful in inhibiting the two most prominent quantitative features of naloxone precipitated withdrawal symptoms - stereotyped jumping and body weight loss. Determination of $IC_{50}$ of S4 revealed a greater affinity towards μ compared to κ receptor. In conclusion, quinoline derivatives of S4 like structure offer potential tool for treatment of narcotic addictions.



## 1. Introduction

Narcotic addiction is perhaps the most common example of drug abuse worldwide. Unlike treatment of other simple or complex diseases, treatment of addiction has a unique disadvantage of high incidence of relapse. Therefore, the approach for the treatment of narcotic addiction, over the years, has been aimed at controlling the withdrawal symptoms that occur after prolonged exposure to the drug of abuse. The first meaningful therapeutic approach towards treatment of opioid addiction was ushered about fifty years ago, by the development of methadone,[1] which is itself a behaviorally selective analgesic. However methadone was beset with the problem of high incidence of relapse when the drug is discontinued, although it was effective while the treatment is maintained. Subsequently, many other compounds like the methadone congener LAAM (levo-alpha-acetyl-methadol), clonidine,[2] L-type calcium channel blockers,[3] NMDA antagonists,[4] Buprenorphine,[5] and naltrexone[6] have been developed and tried for the treatment of narcotic addiction with limited success. Additionally, nitric oxide synthase inhibitors,[7] NMDA blockers[4,8] or recently isoquinoline derivatives[9] have also been investigated for anti-addictive properties.

Since unlike mu-opioid receptors, κ-opioid receptors is not considered to mediate tolerance and dependence several classes of κ-opioid ligands have been explored, such as non peptide κ-agonist benzomorphans (cyclazocine, bremazocine and 8-CAC), arylacetamide (U50488, U69593 and R-84760), epoxymorphinans (nalfurafine), ibogaine (a naturally occurring indole alkaloid). These drugs or kappa ligands effectively modulate/attenuate some of the aspects of addictive substances, such as cocaine, morphine, methamphetamine, etc.[10] Unfortunately, these compounds exhibited behavioral side effects such as sedation, emesis, vomiting[11], raising questions of their uses. Analysis of the effectiveness of κ-agonist for treatment of addiction versus its side effects revealed that, in compounds, which have additional μ agonist/antagonist activity, the side effect is significantly reduced and improvement occurs in their therapeutic efficacy.[12]

There is a growing hypothesis suggesting that a ligand having significant affinity to all three opioid receptors, but with different combinations of agonistic and antagonistic properties at these receptors, may prove to be a promising, behaviorally selective analgesic with diminished side effects.[13] Structural analysis of a series of compounds showing non-specific activity to all three opioid receptors suggested a common 3D pharmacophoric element for the recognition of the three opioid receptors. This common 3D recognition pharmacophore at μ, κ and δ opioid receptors consists of four components—a protonated amine, two hydrophobic groups and the centroid of an aromatic ring- in a geometric arrangement,

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common to all binders at the three opioid receptors. A somewhat similar hypothesis based on Beckett and Casy's 3-point model suggested a structure containing an amine that is thought to be protonated at physiological pH, a phenolic ring and a hydrophobic site in order for the opioid ligand to bind non-selectively to all the three receptor types.[14]

Based on the established 3D pharmacophores for opioid ligands, a series of compounds were synthesized having quinoline motifs. The present study provided the evidence that quinoline compounds are effective to attenuate few features of opiate dependence. Few of these compounds, S4 [$C_{18}H_{17}N_3O$; 2-(2-methylquinolin-4-ylamino)-*N*-phenylacetamide], NS1 [2-(2-methylquinolin-4-ylamino)-*N*-*p*-tolylacetamide], etc., were assessed for their activity on antagonizing drug dependence which was manifested by their ability to control abstinence syndrome following naloxone precipitation in chronically morphine treated mice.

## 2. Results and discussion

### 2.1. Chemistry

Various chloro-*N*-phenylacetamides (S4, PP-2, PP-3, PP-5, NS-1) or chloro-propionamides (PP-7, PP-10) were synthesized by reacting chloroacetyl chloride or chloro propionyl chloride with substituted anilines or cyclohexyl amines (PP-6) or dichloroacetyl chloride with 4-aminoquinaldine. The dichloro ethanone derivative (PP-4) was synthesized by Friedel–Crafts acylation of toluene with dichloroacetyl chloride. These chloro-substituted acetamides or propionamides or ethanones were then condensed with 4-aminoquinaldine (PP-11) in the presence of sodium hydride in dry DMSO under nitrogen atmosphere giving rise to the products (Scheme 1, Table 1). However, for the preparation of PP-4, where the condensation was effected between 4-amino quinaldine and 2,2-dichloro-1-*p*-tolylethanone, an iodoform like reaction took place giving rise to the benzamide derivatives by the elimination of dichloromethane due to the presence of two electron withdrawing chlorine atoms in 2,2-dichloro-1-*p*-tolylethanone (Scheme 2). For the preparation of PP-6 using *N*-cyclohexylchloroacetamide, two units of the acetamide derivative were condensed with 4-aminoquinaldine or PP-11 (Scheme 3). All the compounds were separated by column chromatography over silica gel (60–120 mesh) eluting with different ratio of chloroform–methanol mixture and were characterized by their spectroscopic analysis (IR, $^1H$, $^{13}C$, and MS).

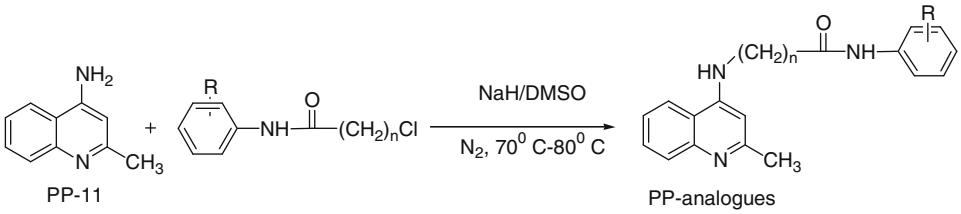
### 2.2. Biology

There have been serious attempts, in the past, to develop compounds having considerable activity at the opiate receptors based on existing knowledge of activities of similar structures. Enhancement of a particular activity (agonist/antagonist) has been exhibited by introducing the functional group 14-alkoxy in *N*-methyl-6-ketomorphinans, which not only enhances its affinity to opioid receptor, but also increases antinociceptive potency of that compound.[15] It is also found that enhancement of the lipophilic properties by substituting functional groups of the opioid antagonists, which are used for the treatment of drug abuse, broadens their therapeutic scope by facilitating their route of administration.[16] The enrichment of structure activity relationship study is also observed in the report of synthesis of a series of homo- and hetero-dimeric ligands containing κ-agonist and μ-agonist/antagonist pharmacophore joined by a linker chain of varying length.[17] Evaluation of the binding affinity of these compounds at μ, κ and δ receptors suggest that the stereochemistry of the pharmacophore, viz. N-substituents, ester linkages and the spacer length were crucial factors for optimum interactions of such ligands at opioid receptor binding sites. Analysis of the binding properties of a series of synthetic dimeric morphinan ligands to three opioid receptors[18] showed a few dimeric structures possessing significant κ agonist and partial μ agonist like its monomeric counterpart while another demonstrated partial agonistic property to both κ and μ receptor.

The quinoline derivative S4 [$C_{18}H_{17}N_3O$; 2-(2-methylquinolin-4-ylamino)-*N*-phenyl acetamide], reported in the present study, affords a structure, which has similar components of the 3D pharmacophore proposed by others,[14] having a side-arm structure, possessing a centroid aromatic ring at the free terminal, a hydrophobic group and a protonated amine. Receptor binding also demonstrated that S4 interacts at the μ- as well as κ-opioid receptor but had no effect at the δ receptor (Table 1). We have also looked at the opioid receptor binding characteristics of similar type of quinolines where the side arm has been modified (Table 1). In addition to the side arm, since all the compounds contained a quinoline moiety, we also looked at the binding characteristics of PP10, having only the side chain structure, and at PP11 and PP12, having only the quinoline core, essentially to establish the contributions of the two basic components in the structure in inducing opioidergic activity.

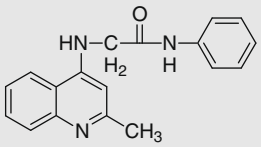
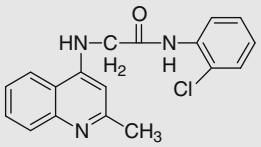
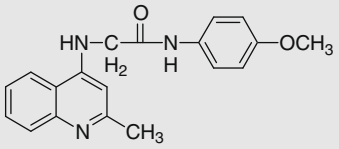
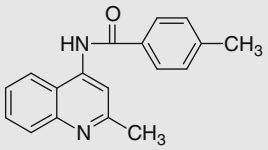
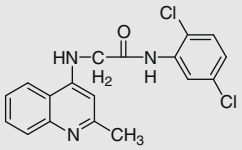
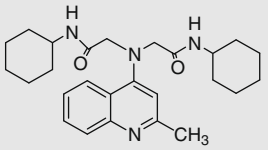
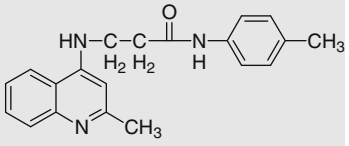
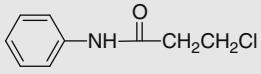
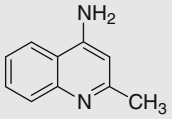
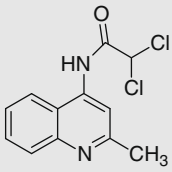
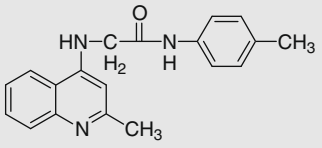
Displacement of specific [$^3H$] DAMGO binding to evaluate the interactions at the μ-opioid receptor showed that all compounds, excepting PP12 and PP11, were able to displace the specific [$^3H$] DAMGO binding. The absence of MOR interaction in both PP11 and PP12 suggested that the quinoline moiety might not contribute towards μ-opioidergic activity. The order of potency of the test compounds in interacting with MOR was as follows: PP7 > PP3 > S4 ≫ NS1 > PP2 > PP6 > PP4 ≈ PP10 > PP5; PP11 and PP12 were inactive.

Displacement of specific [$^{125}I$] Dynorphin binding were carried out with membranes prepared from C6 glioma cells stably transfected with hKOR (human kappa opioid receptor) primarily due to its low tissue binding in rodent brain and also rule out any interaction of dynorphin with nociceptin receptors.[19] While the KOR specific ligand, U50488H, at 10 μM, inhibited specific [$^{125}I$] Dynorphin binding by approximately 80%, the test compounds, with the exception of PP6, exhibited different degree of interaction at KOR binding site at similar concentrations. Among all test compounds, S4 demonstrated the highest displacement of specific binding of [$^{125}I$] Dynorphin binding followed by PP3 and then PP2. Unlike lack of binding at MOR, PP11 and PP12 showed significant interactions at KOR suggesting contributions of the quinoline structure for binding to KOR. On the other hand, the compound PP10, consisting




**Scheme 1.** General reaction procedure for preparation of PP-analogues.

**Table 1**
Yield and structure of the quinoline derivatives synthesized in the laboratory

| Products | Yield (%) | Structure |
|---|---|---|
| S4 | 90 |  |
| PP-2 | 92 |  |
| PP-3 | 90 |  |
| PP-4 | 90 |  |
| PP-5 | 91 |  |
| PP-6 | 88 |  |
| PP-7 | 90 |  |
| PP-10 | 96 |  |
| PP-11 | – |  |
| PP-12 | 94 |  |
| NS1 | 92 |  |

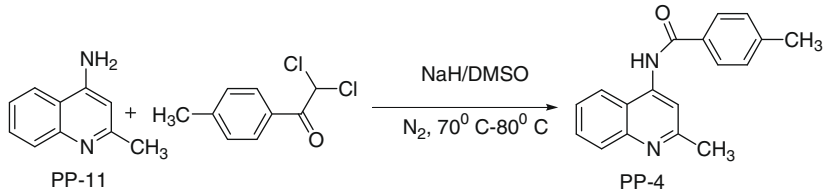
of only the side arm, displayed binding to both KOR and MOR. Moreover, displacement of [$^{125}$I] Dynorphin binding by PP10 was much greater compared to that by PP11 or PP12, suggesting the importance of the side chain for imparting κ-activity in the compounds. PP6, which showed interaction at MOR binding site, had no effect at KOR site. Overall, the order of potency of the test compounds in interacting with KOR was as follows: S4 > PP3 > PP2 > PP7 ≈ PP4 ≈ PP10 > PP11 > PP5 ≈ PP12 > NSI; PP6 was inactive (Table 2).

On the contrary, studies on the interaction of the test compounds at δ-opioid receptor showed that none of the compounds, with the exception of PP7, had any appreciable effect in displacing specific [$^{125}$I] DADLE binding. Surprisingly, PP7 increased specific [$^{125}$I] DADLE binding by about 70%. Affinity studies for the interaction at the μ- and κ-opioid receptors by S4 were evaluated by calculating the $IC_{50}$ values. It was observed that S4 had high affinity for both μ- and κ-opioid receptors, which was comparable to that of selective ligands like DAMGO and U50488H for the respective receptors (Fig. 1).

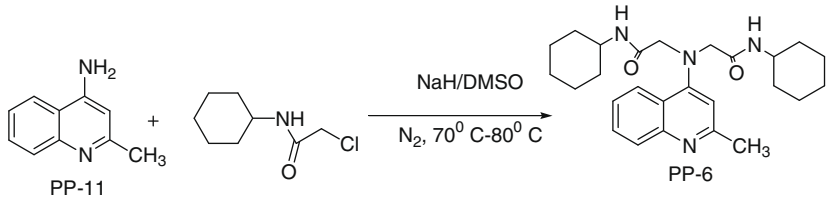
Overall, results from the binding studies suggest a vital influence of the side-arm structure as well as its linkage with the nitrogen-containing ring of quinoloine in imparting opioidergic activity in the compounds. Of the three essential components of the side-arm structure, it appears that the presence of phenyl ring (without any substitution) at the free end of the chain and also free rotation of this phenyl ring is vital for KOR binding activity since S4 has a greater activity than other compounds such as PP3, PP2, etc. The importance of the terminal phenyl ring structure in S4 can be explained briefly by comparing the same in other compounds, such as PP2, PP3. The compounds PP2 and PP3 have almost similar structures, differing only in the substitution in the phenyl ring with –Cl at ortho position for PP2 and –OMe at para position for PP3. The comparatively lower activity of PP2 at the κ receptor may be due to the presence of –Cl at ortho position causing steric hindrance hereby the terminal phenyl ring is unable to remain in the same plane with the rest of the side chain. Considering the structure of PP3 compound, due to the +I effect of –OMe group on the phenyl ring, that is, the electron donating property, raised the possibility of creating a partial double bond between the phenyl ring and nitrogen atom adjacent to the phenyl ring resulting in restricted rotation of terminal phenyl ring. Interestingly, the importance of free rotation of terminal phenyl ring has been observed for a number of known nitrogenous KOR ligand including U69593, and U50488H.

For binding to μ-opioid receptors, the phenyl ring of the side-arm structure also appeared to be important for the tested compounds. It was also supported from the data of PP12, which has a side chain where the Ph ring is absent, was unable to bind to MOR. A number of μ-specific compounds like morphine, codeine and heroin also possess benzene ring with substitutions by –OH, $–OCH_3$ and $–OCOCH_3$, respectively. In such cases the benzene ring is restricted in the space, due to its bonding to other components in the structure. Thus unlike, KOR ligands, free rotation does not appear to be essential for MOR binding. Even, higher degree of interaction of PP3 and PP7 to MOR, compared to S4 could be due to the presence of restricted rotation of terminal phenyl ring.

Both κ- and μ-opioid receptor agonists and not antagonist's causes transient activation on pERK[20] in cultured cells within a few minutes of application. Receptor specific antagonists also inhibited such stimulatory effect on pERK by the opioid agonists. The above observations were applied in primary cultures of astrocytes with a view to determine the opioid agonistic or antagonistic property of the test compounds. Astrocyte cultures were treated with the test compounds for 10 min and pERK levels were estimated by western blotting analysis. Like the κ-opioid ligand, U50488H and the μ-specific ligand DAMGO (data not shown), all the tested compounds were able to stimulate ERK phophorylation




**Scheme 2.** Preparation of PP-4.




**Scheme 3.** Preparation of PP-6.

**Table 2**
Effect of quinoline compounds on the specific bindings of μ, κ and δ opioid receptors

| | Specific binding (fmol/mg protein) | | |
|---|---|---|---|
| Compound | μ | κ | δ |
| – | 6.40 ± 0.25 (100) | 57.46 ± 1.11 (100) | 10.91 ± 0.45 (100) |
| DAMGO | 4.86 ± 0.19 (76) | | |
| U50488H | | 10.63 ± 0.20 (19) | |
| Naloxone | | | 2.50 ± 0.10 (23) |
| S-4 | 1.92 ± 0.07 (30) | 21.83 ± 0.42 (38) | 10.72 ± 0.44 (98) |
| PP-2 | 3.30 ± 0.13 (52) | 29.88 ± 0.57 (52) | 7.90 ± 0.33 (72) |
| PP-3 | 1.7 ± 0.06 (27) | 25.28 ± 0.48 (44) | 7.96 ± 0.33 (73) |
| PP-4 | 3.95 ± 0.15 (62) | 33.33 ± 0.64 (58) | 9.16 ± 0.38 (85) |
| PP-5 | 4.20 ± 0.16 (66) | 42.52 ± 0.82 (74) | 10.47 ± 0.43 (96) |
| PP-6 | 3.42 ± 0.13 (53) | 65.51 ± 1.26 (114) | 10.80 ± 0.45 (99) |
| PP-7 | 1.45 ± 0.05 (23) | 32.18 ± 0.62 (56) | 17.01 ± 0.71 (156) |
| PP-10 | 3.91 ± 0.15 (61) | 33.9 ± 0.65 (59) | 10.80 ± 0.45 (99) |
| PP-11 | 6.08 ± 0.23 (95) | 40.8 ± 0.78 (71) | 10.47 ± 0.43 (96) |
| PP-12 | 6.78 ± 0.26 (106) | 43.67 ± 0.84 (77) | 10.25 ± 0.42 (94) |
| NS-1 | 3.01 ± 0.11 (47) | 51.71 ± 0.99 (90) | 10.47 ± 0.43 (97) |

Binding at μ , κ and δ opioid receptors were carried out using $^{3}$H-DAMGO, $^{125}$I-Dynorphin and $^{125}$I- DADLE, respectively, in absence and presence of the test compounds. For displacement of $^{3}$H-DAMGO, concentrations of test compounds were 5 nM while for $^{125}$I- Dynorphin and $^{125}$I- DADLE, 5 μM of test compound was used. binding at each type of receptor was determined from the difference in binding in absence and presence of 0.05 nM of unlabeled DAMGO (μ) or 50 μM of U50488H (κ) or 100-μM naloxone (δ). Specific displacement of test compounds at μ, κ and δ opioid receptors were compared with that by equal concentrations of unlabeled DAMGO, U50488H and naloxone, respectively. $^{3}$H-DAMGO and $^{125}$I-DADLE binding was carried out in mouse brain membrane while $^{125}$I- Dynorphin binding was undertaken in membranes prepared from of C6 cell line stably transfected with κ opioid receptor. Details of methodology are described in Materials and Methods. Data represent mean ± SEM from three experiments. Values within parenthesis represent percentage binding compared to total binding in absence of any test compound, which is taken as 100.

(Fig. 2a, b and c). Stimulation of pERK1/2 level by the test compound was also sensitive to the opioid receptor antagonist naloxone (10 μM), which could effectively block the stimulation but by itself had no effect (Fig. 2a).

Having observed that all of the test compounds exhibited opioid agonistic properties with varying degree of interaction at the μ- and κ-opioid receptors, we evaluated by the ability of the compounds to check physical dependence during naloxone precipitated abstinence in mice chronically treated with morphine. While animals were scored for all the vital signs of abstinence, such as writhing, wet dog shake, grooming, diarrhea, etc., these were not quantified for this study. We have reported the effect of the test compounds on stereotype jumping as well as body weight loss that is considered to be quantitatively related to abstinence.[21] The selection of the tested compounds for withdrawal testing was done to some extent on the basis of their interaction to KOR and MOR. Since both PP4 and PP2 had almost equal interaction at two receptors, results could provide information on the contribution of equal interactions on morphine dependence. Since MOR specific ligands are better in antagonizing physical dependence during opioid withdrawal, we selected PP7 and NS1. PP7 had greater binding to MOR than KOR, where as NS1 had binding mainly to MOR. PP10 was selected to find the contribution of the side chain of S4 compound in withdrawal.

The graphical representation of stereotype jumping during naloxone precipitated withdrawal in absence and presence of the test compounds are presented in Figure 3. It was observed that chronic morphine treated mice when challenged with naloxone (10 mg/kg) caused an average of six jumps within the 20 min period of observation. Daily injection of a single dose (20 mg/kg) of S4, PP2, PP4 or NS1, throughout the six days of chronic morphine treatment could completely inhibit jumping response whereas similar treatment with PP7, PP10 decreased the incidence of stereotype jumping to a small extent. However, a single injection of S4, just before naloxone challenge, had no effect on stereotype jumping.

Physical dependence was also verified by the loss in body weight following precipitated withdrawal. Graphical representation of body weight loss in absence and presence of the test compounds are represented in Figure 4. Naloxone precipitated abstinence in mice caused a significant loss in body weight. S4 (20 mg/kg), at both chronic and acute doses, inhibited this loss in body weight. Likewise, daily injection of a single dose (20 mg/kg) of PP4 or PP10 for 6 days could also attenuate naloxone precipitated body weight loss significantly.

Interestingly, our results on the effect of the test compounds on precipitated withdrawal failed to provide any correlation with the structure of the compounds or the selective contributions of MOR and KOR, based on our binding studies. It is likely that these two prominent withdrawal features of stereotype jumping and weight loss may have other independent controlling systems. A review of the literature in support of our inference is evident from the report that NalB, a naloxone derivative and a putative antagonist of nociceptive receptor, reduced the frequency of jumping in nociceptin receptor knockout mice compared to wild type, although the other withdrawal behaviour such as forepaw tremor, rearning were identical in both mice.[22] Intrathecal administration (i.t) of U50488H attenuated three (wet shakes, escaped attempts, teeth chattering) out of four withdrawal signs, in naloxone-precipitated

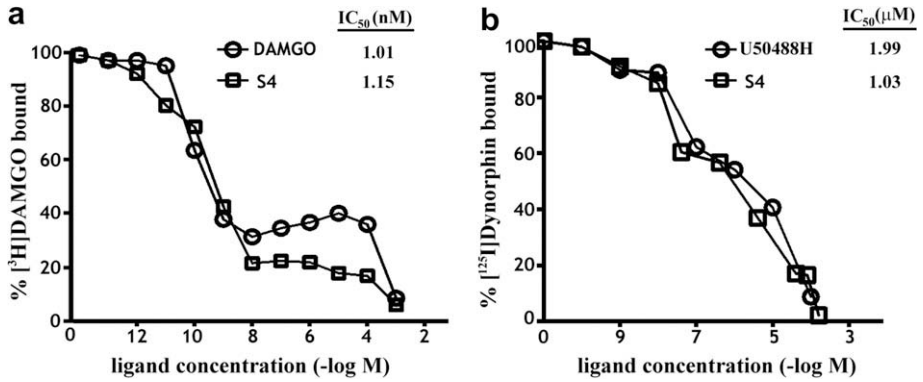



**Figure 1.** Competitive inhibition of specific (a) [$^3$H]DAMGO and (b) [$^{125}$I]Dynorphin binding to brain membrane by S4. Corresponding displacement curves by selective agonists, DAMGO (a) and U50488H (b) were also carried out for comparison. Each point represents mean of three individual determinations. Non-linear regression lines were determined by Graph prism 5 and $IC_{50}$ concentrations calculated.

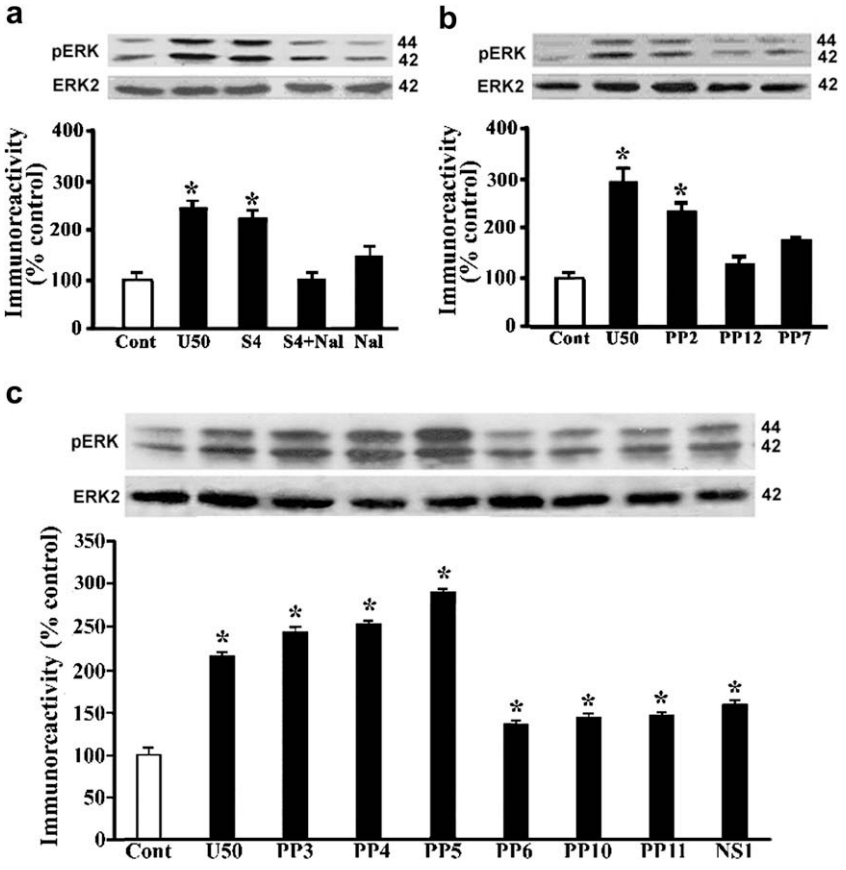



**Figure 2.** Effect of test compounds on induction of pERK activity in primary cultures of astrocytes. Ten-day-old cultures were serum starved for 48 h. The stimulatory effect of opioid agonist such as κ-opioid receptor agonist, U50488H (1 μM) and the test compounds (40 μM) were evaluated by treating cells for 10 min with the drugs and quantitating pERK1/2 by western blot analysis. a) Shows stimulation of pERK by S4, which was antagonized by co-treatment with naloxone (10 μM). The effect of other test compounds on pERK activation includes PP2, PP12, PP7 in (b) and PP3, PP4, PP5, PP6, PP10, PP11, NS1 in (c). Same blots were probed with ERK2 which served as a loading control. The relative intensities of the pERK bands, indicated in the graph, were obtained by densitometric scanning and pERK levels were quantitated after normalizing against ERK2. Results are mean ± SEM of at least 3 blots. $^*P$ <0.05 versus untreated control.

withdrawal[23] but was unable to attenuate weight loss. The κ-selective antagonist nor-BNI, unlike U50488H, potentiated four withdrawal signs differentially in this study. Nor-BNI significantly aggravated body weight loss, teeth chattering, wet shakes, but escaped attempts were not markedly affected compare to control. In the present study, none of our test compounds potentiated withdrawal signs possibly due to their agonistic property at KOR as evident from our observations of their stimulatory effect on pERK.

## 3. Conclusion

Based on present concepts of drug designing, the present study is an attempt towards developing potential drugs having mixed opioidergic property and capable of antagonizing physical dependence. A number of quinoline like compounds having typical pharmacophore for opioidergic activity have been tested and our observations suggest a potential therapeutic use of quinoline like

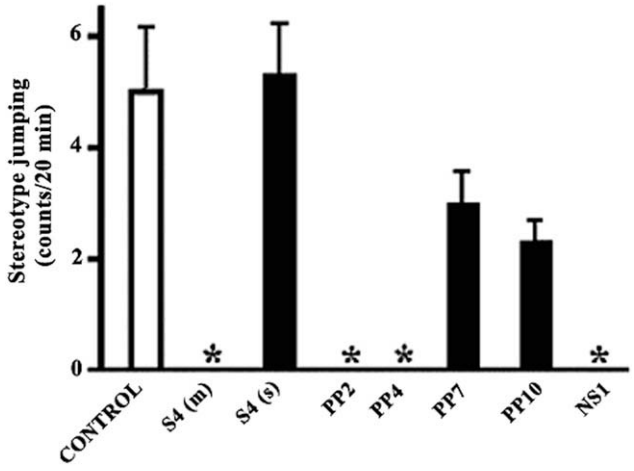



**Figure 3.** Demonstration of physical dependence by naloxone-precipitated stereotype jumping. Mice (seven per group) in each experiment were treated with chronic morphine regime. Treatment protocol for test compounds induction of precipitated withdrawal by naloxone is described in Methods. S4 (S) denotes S4 injected singly after the last dose of morphine. Otherwise, test drugs were given once daily for 6 days. Stereotype jumping in each mouse was scored within a 20-min period after each injection. Data are expressed as mean ± SE of stereotyped jumping in counts per 20 min of three individual experiments * indicates significantly different ($p$ <0.001) from control.

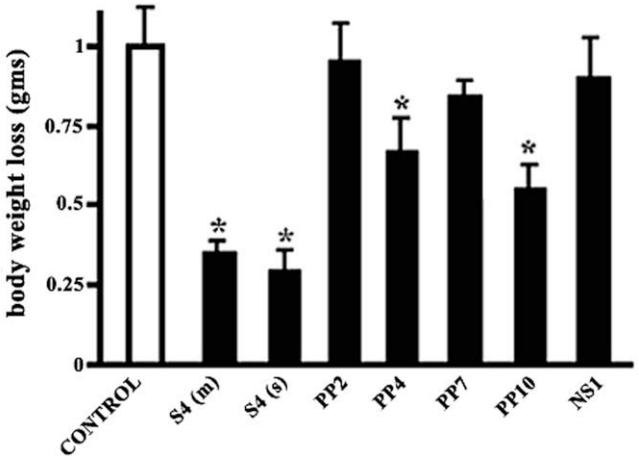



**Figure 4.** Effect of naloxone precipitated withdrawal on body weight of mice. Mice (seven per group) in each experiment were treated with chronic morphine regime. Treatment protocol for test compounds and induction of precipitated withdrawal by naloxone are described in Methods. S4 (S) denotes S4 injected singly after the last dose of morphine. Otherwise, test drugs were given once daily for 6 days. Data are expressed as mean ± SE of Body weight loss before naloxone injection and 4 h after of three individual experiments * indicates significantly different ($p$ <0.05) from control.

compounds in the management of the opiate tolerance and withdrawal syndrome.

## 4. Experimental

### 4.1. Chemistry

#### 4.1.1. General methods

Melting points were determined with a capillary melting point apparatus and are uncorrected. IR spectra were recorded on a JASCO FTIR (model 410) in KBr pellets. ESI-MS and HRMS (positive) was conducted using LC-ESI-Q-TOF micro Mass spectrometer. $^1$H and $^{13}$C NMR spectra were taken on a Bruker 300 MHz DPX spectrometer at 300 and 74.99 MHz, respectively, with tetramethylsilane as internal standard and the chemical shifts are reported in $\delta$ units. 4-Aminoquinalidine and sodium hydride were purchased from Aldrich Chemical Ltd (USA). Organic solvents used for the chemical synthesis and for chromatography acquired from E. Merck (India) were of analytical grade. All chromatographic purification was performed with silica gel (60–120 mesh) and was obtained from SRL (India). Thin layer chromatography was performed on pre-coated silica gel 60 $F_{254}$ aluminum sheets (E. Merck, Germany) using the solvent system 1–6% MeOH in $CHCl_3$ and spots were developed using Liebermann–Burchard solution.

#### 4.1.2. General method of preparation of PP analogues

The preparation of each PP analogue was carried out in a three-necked round bottom flask. The whole system was placed on a magnetic stirrer with a magnetic fly inside the flask. Sodium hydride was taken in the flask and was washed with dry non-polar solvent petroleum ether to make it free from suspended oil. Dry dimethyl sulphoxide was added and the reaction mixture was stirred (about 20 min) and heated until it reaches at around 40 °C. The substrate (4-aminoquinalidine) was added to the reaction mixture and stirring continued for another 20 min. Finally, the reactant (mono chloro-anilides) was added to the reaction mixture and stirring continued for another 8 h at 70–80 °C. The whole operation was carried out in an inert atmosphere, viz. nitrogen, argon free from oxygen and in anhydrous condition. Termination of the reaction was accomplished by adding ice-cold water (taking care that the temperature of the reactants did not go beyond 4–10 °C). The whole of the reaction mixture was extracted with ethyl acetate. The organic layer was washed with water to make it free from alkali (neutral to litmus). The organic solvent was removed under reduced pressure to furnish PP analogues after crystallization from methanol.

#### 4.1.3. 2-(2-Methylquinoline-4-ylamino)-*N*-phenylacetamide

See Ref. 24.

#### 4.1.4. NS-1: 2-(2-Methylquinoline-4-ylamino)-*N*-*p*-tolylacetamide

Obtained as white solid, mp 228–230 °C; IR (KBr, $\nu_{max}$) 3410, 1682, 1529, 1324 cm$^{-1}$; $^1$H NMR (DMSO-$d_6$) $\delta$ 2.25 (s, 3H, –$CH_3$), 2.44 (s, 3H, –$CH_3$), 4.13 (d, 2H, $J$ = 5.2 Hz, –$CH_2$), 6.29 (s, 1H, –$NHCH_2$) 7.12 (d, 2H, $J$ = 7.8 Hz), 7.37–7.51 (m, 4H), 7.59 (m, 1H), 7.73 (d, 1H, $J$ = 8.4 Hz), 8.16 (d, 1H, $J$ = 8.1 Hz), 10.08 (s, 1H, –CONH).

$^{13}$C NMR (DMSO-$d_6$) $\delta$ 21.3 (–$CH_3$), 26.1 (–$CH_3$), 47.1 (–$CH_2$), 99.4 (–CH), 118.4 (–C), 120.3 (2 × –CH), 122.3 (–CH), 124.2 (–CH), 129.2 (–CH), 129.7 (–CH), 130.0 (2 × –CH), 133.2 (–C), 137.1 (–C), 148.8 (–C), 151.0 (–C), 159.4 (–C), 168.7 (–C=O); MS (ESI-MS, positive ion) $m/z$ 306 $[M+H]^+$; HRMS $m/z$ $[M+H]^+$ 306.1562 [calcd 306.1606].

#### 4.1.5. PP-2: *N*-(2-Chlorophenyl)-2-(2-methylquinoline-4-ylamino)acetamide

Obtained as white solid, mp 177–179 °C; IR (KBr, $\nu_{max}$) 3250, 1674, 1530, 1436 cm$^{-1}$; $^1$H NMR (DMSO-$d_6$) $\delta$ 2.50 (s, 3H, –$CH_3$), 4.20 (d, 2H, $J$ = 5.1 Hz, –$CH_2$), 6.36 (s, 1 H, –$NHCH_2$), 7.20 (t, 1H, $J$ = 7.8 Hz), 7.34 (t, 1H, $J$ = 7.8 Hz), 7.41 (t, 1H, $J$ = 7.8 Hz), 7.49 (d, 1H, $J$ = 7.8 Hz), 7.57–7.62 (m, 2H), 7.76 (t, 2H, $J$ = 8.4 Hz), 8.18 (d, 1H, $J$ = 8.4 Hz), 9.71 (s, 1H, –CONH).

$^{13}$C NMR (DMSO-$d_6$) $\delta$ 25.2 (–$CH_3$), 46.2 (–$CH_2$), 98.9 (–CH), 117.6 (–C), 121.4 (–CH), 123.4 (–CH), 125.7 (–CH), 126.4 (–C), 126.5 (–CH), 127.6 (–CH), 128.3 (–CH), 128.9 (–CH), 129.5 (–CH), 134.5 (–C), 148.0 (–C), 150.0 (–C), 158.6 (–C), 168.7 (–C=O); MS (ESI-MS, positive ion) $m/z$ 326$[M+H]^+$; HRMS $m/z$ $[M+H]^+$ 326.1085 [calcd 326.1060].

#### 4.1.6. PP-3: *N*-(4-Methoxyphenyl)-2-(2-methylquinoline-4-ylamino)acetamide

Obtained as white solid, mp 216–218 °C; IR (KBr, $\nu_{max}$) 3407, 1675, 1536, 1244 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 2.44 (s, 3H, $-CH_3$), 3.71 (s, 3H, $-OCH_3$), 4.11 (d, 2H, $J$ = 5.7 Hz, $-CH_2$), 6.29 (s, 1H, $-NHCH_2$), 6.89 (d, 2H, $J$ = 8.7 Hz), 7.37–7.53 (m, 4H), 7.59 (t, 1H, $J$ = 7.8 Hz), 7.73 (d, 1H, $J$ = 8.1 Hz), 8.16 (d, 1H, $J$ = 8.1 Hz), 10.04 (s, 1H, –CONH).

$^{13}C$ NMR (DMSO-$d_6$) $\delta$ 26.1 ($-CH_3$), 47.0 ($-CH_2$), 56.0 (–OMe), 99.5 (–CH), 114.8 (2 × –CH), 118.4 (–C), 121.9 (2 × –CH), 122.3 (–CH), 124.2 (–CH), 129.1 (–CH), 129.7 (–CH), 132.7 (–C), 148.8 (–C), 151.1 (–C), 156.3 (–C), 159.4 (–C), 168.5 (–C=O); MS (ESI-MS, positive ion) $m/z$ 322 $[M+H]^+$; HRMS $m/z$ $[M+H]^+$ 322.1540 [calcd 322.1556].

#### 4.1.7. PP-4: 4-Methyl-*N*-(2-methylquinoline-4-yl)benzamide

Obtained as white solid, mp 137–139 °C; IR (KBr, $\nu_{max}$) 3297, 1659, 1522, 1282 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 2.42 (s, 3H, $-CH_3$), 2.67 (s, 3H, $-CH_3$), 7.39 (d, 2H, $J$ = 7.8 Hz), 7.55 (m, 1H), 7.73 (m, 1H), 7.83 (s, 1H), 7.97 (m, 3H), 8.17 (d, 1H, $J$ = 8.1 Hz), 10.52 (s, 1H, –CONH).

$^{13}C$ NMR (DMSO-$d_6$) $\delta$ 21.9 ($-CH_3$), 26.0 (–C–$CH_3$), 116.4 (–CH), 121.9 (–C), 123.9 (–CH), 125.9 (–CH), 129.0 (2 × –CH), 129.4 (–CH), 129.9 (2 × –CH), 130.3 (–CH), 132.3 (–C), 142.9 (–C), 143.1 (–C), 149.3 (–C), 159.7 (–C), 167.2 (–C=O); MS (ESI-MS, positive ion) $m/z$ 277 $[M+H]^+$; HRMS $m/z$ $[M+H]^+$ 277.1317 [calcd 277.1341].

#### 4.1.8. PP-5: *N*- (2,5-Dichlorophenyl)-2-(2-methylquinoline-4-ylamino)acetamide

Obtained as white solid, mp 215–217 °C; IR (KBr, $\nu_{max}$) 3197, 1676, 1529, 1255 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 2.46 (s, 3H, –C–$CH_3$), 4.24 (d, 2H, $J$ = 5.4 Hz, $-CH_2$), 6.36 (s, 1H, $-NHCH_2$), 7.26 (dd, 1H, $J$ = 2.4 Hz, 8.7 Hz), 7.41 (t, 1H, $J$ = 7.8 Hz), 7.53 (d, 1H, $J$ = 8.7 Hz), 7.60 (m, 2H), 7.74 (d, 1H, $J$ = 8.4 Hz), 7.94 (d, 1H, $J$ = 2.4 Hz), 8.17 (d, 1H, $J$ = 8.1 Hz), 9.81 (s, 1H, –CONH).

$^{13}C$ NMR (DMSO-$d_6$) $\delta$ 25.2 ($-CH_3$), 46.2 ($-CH_2$), 98.9 (–CH), 117.6 (–C), 121.4 (–CH), 123.5 (–CH), 124.3(–C), 124.4 (–CH), 125.9 (–CH), 128.3 (–CH), 128.9 (–CH), 130.8 (–CH), 131.7 (–C), 135.8 (–C), 147.9 (–C), 149.9 (–C), 158.6 (–C), 169.0 (–C=O); MS (ESI-MS, positive ion) $m/z$ 360 $[M+H]^+$, 362$[M+2+H]^+$; HRMS $m/z$ $[M+H]^+$ 360.0688 [calcd 360.0670].

#### 4.1.9. PP-6: *N*-Cyclohexyl-2-[cyclohexylcarbamoylmethyl-(2-methylquinoline-4-yl)-amino]acetamide

Obtained as white solid, mp 230–232 °C; IR (KBr, $\nu_{max}$) 3273, 2930, 1653, 1431, 1245 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 1.05–1.17 (m, 6H), 1.26–1.38 (m, 4H), 1.54–1.65 (m, 6H), 1.79–1.83 (m, 4H), 2.63 (s, 3H, $-CH_3$), 3.74–3.84 (m, 2H), 3.96 (s, 4H), 6.82 (s, 1H), 6.94 (d, 2H, $J$ = 8.1 Hz), 7.41(t, 1H, $J$ = 7.5 Hz), 7.62 (t, 1H, $J$ = 7.8 Hz), 7.98 (t, 2H, $J$ = 8.1 Hz, –CONH).

$^{13}C$ NMR ($CDCl_3$) $\delta$ 25.0 (4 × $-CH_2$), 25.7 (2 × $-CH_2$), 25.9 ($-CH_3$), 33.2 (4 × $-CH_2$), 48.7 (2 × –CH), 58.3 (2 × $-CH_2$), 109.9 (–CH), 121.7 (–C), 123.1 (–CH), 125.4 (–CH), 129.77 (–CH), 129.80 (–CH), 150.1 (–C), 153.7 (–C), 159.4 (–C), 168.4 (2 × –C=O); MS (ESI-MS, positive ion) $m/z$ 437 $[M+H]^+$; HRMS $m/z$ $[M+H]^+$ 437.2887 [calcd 437.2917]; $[M+Na]^+$ 459.2701 [calcd 459.2736].

#### 4.1.10. PP-7: 3-(2-Methylquinoline-4-ylamino)-*N*-*p*-tolylpropionamide

Obtained as white solid, mp 241–243 °C; IR (KBr, $\nu_{max}$) 3365, 1668, 1542, 1351 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 2.24 (s, 3H, $-CH_3$), 2.47 (s, 3H, $-CH_3$), 2.73 (t, 2H, $J$ = 6.6 Hz, $-CH_2$), 3.60 (t, 2H, $J$ = 6.0 Hz, $-CH_2$), 6.44 (s, 1H, $-NHCH_2$), 7.09 (d, 2H, $J$ = 8.1 Hz), 7.16 (m, 1H), 7.33 (t, 1H, $J$ = 7.5 Hz), 7.48–7.58 (m, 3H), 7.70 (d, 1H, $J$ = 8.1 Hz), 8.12 (d, 1H, $J$ = 8.4 Hz), 9.96 (s, 1H, –CONH).

$^{13}C$ NMR (DMSO-$d_6$) $\delta$ 21.3 ($-CH_3$), 26.0 ($-CH_3$), 36.3 ($-CH_2$), 39.5($-CH_2$), 99.1 (–CH), 118.4 (–C), 120.1 (2 × –CH), 122.2 (–CH), 124.0 (–CH), 129.1 (–CH), 129.6 (–CH), 129.9 (2 × –CH), 132.9 (–C), 137.5 (–C), 148.8 (–C), 150.7 (–C), 159.6 (–C), 170.3 (–C=O); MS (ESI-MS, positive ion) $m/z$ 320 $[M+H]^+$; HRMS $m/z$ $[M+H]^+$ 320.1729 [calcd 320.1763].

#### 4.1.11. PP-10: 3-Chloro-*N*-phenylpropionamide

Obtained as white solid, mp 102–104 °C.

#### 4.1.12. PP-11: 2-Methylquinoline-4-ylamine

Purchased from Aldrich Chemical Ltd (USA).

#### 4.1.13. PP-12: 2,2-Dichloro-*N*-(2-methylquinoline-4-yl)acetamide

Obtained as white solid, mp 80–82 °C.

### 4.2. Biological studies

#### 4.2.1. Materials

DMEM, F12 and FBS were obtained from Gibco-BRL, Life Technologies. Trypsin, soybean trypsin inhibitor, poly-L-lysine hydrobromide (molecular weight >300,000), U50488H, Naloxone hydrochloride, Dynorphin, anti-mouse IgG-HRP conjugated 2nd antibody were from Sigma Chemical Co. (USA). Primary antibody of pERK1/2 from Santacruz (USA), Lipofectamine 2000, G418S from Invitrogen (USA) were purchased. hKOR-pcDNA was from UMR cDNA Resource Centre, University of Missouri-Rolla and C6 cell line from National Centre for Cell Sciences, Pune. [$^3H$] DAMGO, $NaI^{125}$ were from PerkinElmer (Boston, MA). Morphine sulphate was procured from Gluconate India with the permission of the Excise Directorate, Government of West Bengal. All other reagents were of analytical grade and obtained locally.

#### 4.2.2. Animals

The experimental protocols using animals have been approved by the Institutional Animal Ethics Committee and meet the guidelines of the Government of India. For binding experiments as well as for studies on morphine withdrawal, adult albino Balb/c mice, 20–30 g were used. Animals were housed seven per cage at room temperature and allowed to adapt to laboratory conditions for at least 2 days before the initiation of any experiment. The animals were housed under a standard light dark cycle with free access to food and water, except during testing.

#### 4.2.3. Cell culture and stable transfection

C6 cells were transfected with human κ-opioid receptor in pcDNA using Lipofectamine. Stably transfected G418-resistant cells were grown in F12 medium containing 10% fetal calf serum, 50 μg/ml gentamicin, pen-strep and 50 μg/ml G418 in 5% $CO_2$ at 37 °C.

#### 4.2.4. Membrane preparation

For opioid receptor binding studies, membranes were prepared from both the stably transfected cell line (for κ-opioid receptor binding) and from mouse brain (μ-opioid receptor binding). Stably transfected C6 glioma, as described above, were harvested in ice-cold phosphate-buffered saline and homogenized in 50 mM Tris–HCl, pH 7.4. The total membrane (TM) fraction was collected upon centrifugation at 30,000*g* for 20 min.

In case of membrane from mouse brain, mice was sacrificed by decapitation, brain dissected out and homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4) followed by centrifugation at 20,000 rpm for 30 min. The pellet was resuspended in the same buffer and incubated for 20 min at 37 °C followed by centrifugation as above. The pellet was resuspended in ice-cold buffer and used for

binding assay. Protein concentration was determined by the method described by[25] with bovine serum albumin used as standard.

#### 4.2.5. Iodination of Dynorphin

For κ-opioid receptor binding experiments, dynorphin (DPDYN) was labeled with $^{125}$I-sodium iodide by chloramines T method.[26] Briefly, the reaction was initiated when 10 μl of chloramines T (2 mg/ml) dissolved in a 0.2 M phosphate buffer (pH 7.4) was added to a vial containing 10 μl of the compound (1 mg/ml) dissolved in 0.2 M phosphate buffer, 10 μl 0.2 M phosphate buffer and 1 mCi of $Na^{125}I$. After 40 sec of reaction, 40 μl of sodium bisulfite (2 mg/ml) was added. The reaction mixture was diluted to 8 ml with 0.1% aqueous trifluoroacetic acid (TFA) and absorbed in Sep-Pak. After rinsing with 20 ml of 0.1% aqueous TFA, the mixture of labeled and unlabeled compound were eluted out in a 1 ml solution containing 99.9% acetonitrile with 0.1% TFA. Finally, the labeled compound was separated from the unlabeled compound by reverse phase high performance liquid chromatography on a C18 column with elution with a mobile phase of 20:0.2:79.8 acetonitrile/TFA/water. Fractions corresponding to monoiodinated compound were pooled and used for subsequent studies.

#### 4.2.6. Radioligand binding

As described earlier, KOR binding[27] was assayed in the membrane fraction of C6 cells using [$^{125}$I] Dynorphin while MOR and DOR binding[28] assay was carried out in mouse membrane using [$^{3}$H] DAMGO and [$^{125}$I] DADLE, respectively. For KOR binding, membranes (100–200 μg of protein) were incubated with 2 nM [$^{125}$I] Dynorphin for 30 min at 37 °C in 1 ml of 50 mM Tris–HCl buffer, pH 7.4. Nonspecific binding was determined in the presence of 10-μM concentration of unlabeled U-50, 488H. For MOR binding, membrane homogenates (300 μg protein) were incubated for 2 h at 25 °C in 1 ml of 50 mM Tris–HCl (pH 7.4) containing 2 nM [$^{3}$H] DAMGO in presence and absence of cold naloxone to determine specific binding. Following incubation, bound radioligand was collected by filtering under vacuum in a Millipore filtration manifold using glass-fiber filters (GF/B; Whatman, Clifton, NJ), pretreated with 0.5% PEI. The filters were washed thrice with ice-cold buffer and the radioactivities retained on filters were counted in a liquid scintillation counter (Wallac, model 1409–411, Perkin Elmer, USA).

#### 4.2.7. Evaluation of morphine withdrawal

Development of morphine dependence was assessed from naloxone-precipitated withdrawal. Mice were made tolerant to and dependent on morphine by receiving morphine sulfate subcutaneously thrice daily for six consecutive days with an increment of 5 mg/kg/day with an initial starting dose of 10 mg/kg. Morphinized animals were divided into groups. Test compounds were dissolved in DMSO and each group received a particular test compounds which was injected subcutaneously 30 min before morphine injection, once per day except that one group received a single acute dose S4. Control groups received vehicle instead of the test compound. Dosages of the drugs were selected on the basis of previous result obtained from chronic S4 treatment, that is, 20 mg/kg. On day 6, a single dose of morphine was given and 6 h later naloxone (10 mg/kg) was injected ip, whereas tested compounds were given almost 2 h before naloxone injection. Animals were weighed prior to and 4 h after giving naloxone. Immediately after giving naloxone few abstinence syndromes were recorded over a 20-min period.

Physical dependence was measured by quantitation of naloxone-precipitated jumping of individual animals using a PlexiglasR withdrawal chamber over a period of 20 min after naloxone treatment and then the average number of jumps per group was determined. Physical dependence was also evaluated by measuring mouse body weight loss after 4 h of administration of naloxone. Animals were also observed for other signs and symptoms associated with opioid withdrawal (including 'biting', 'wet dog shakes', 'digging', 'face washing', 'grooming', 'rearing', 'scratching', 'diarrhea', and 'ptosis').

#### 4.2.8. Primary cultures of astrocytes from rat brain

Primary cultures of glial cells were prepared from the neocortex of newborn rat pups after cervical dislocation (<24 h old) as reported earlier.[29] Briefly, single-cell suspensions were rinsed with Dulbecco's modified Eagle's medium and then suspended in the same buffer supplemented with 50 μg/ml gentamycin, 50 μg/ml streptomycin, sodium bicarbonate and 10% fetal bovine serum, pH 7.2.[30] Cells were initially plated in 90 mm poly L-lysine hydrobromide (MW >300,000; Sigma)-coated tissue culture plates and kept for 5 min, for preferential attachment of neurons. Two such attachments yielded a highly purified (>95%) preparation of astrocytes. Unattached cells were seeded onto poly L-lysine-coated plates at $6 \times 10^6$ cells per plate. Cultures were maintained in a Forma $CO_2$ incubator (5% $CO_2$, 95% air) at 37 °C for 10 days. For ERK assay cells were maintained additionally 48 h in serum free medium.

#### 4.2.9. ERK assay

ERK phosphorylation was measured by immunoblotting as described.[31] Following starvation for 48 h, cells were treated as indicated. Antagonists were added to the medium 1 h before stimulation with agonist and compounds. After the indicated stimulation period, medium was removed, and plates were washed with ice cold PBS. Cell lysates were collected in lysing buffer (20 mM HEPES, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM sodium vanadate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, and 20 mg/ml leupeptin, pH 7.5). Cell lysates were centrifuged at 14,000g for 20 min at 4 °C, and protein concentration of the supernatants was determined. Samples (40 μg of protein/lane) were separated by 10% SDS–PAGE. Proteins were blotted on PVDF membranes (Millipore Corp., Bedford, MA). Ponceau staining of blots was carried out, prior to immunoblotting, to confirm loading of equal amount of proteins in the lanes. Non-specific binding to membranes was blocked with 5% non-fat dry milk in Tris-buffered saline (20 mM Tris, 0.9% NaCl, pH 7.5) for 1 h at 37 °C. The membranes were then probed with monoclonal antibody against *p*-ERK (1:1000 dilution) for 2–3 h, followed by staining with a peroxidase conjugated secondary antibody.

### 4.3. Statistical analysis

All results are expressed as means ± S.E.M. The data were analyzed by one-way analysis of variance ANOVA followed by Duncan's new multiple range test. Differences with $P < 0.05$ between experimental groups at each point were considered statistically significant.

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