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Original article

Synthesis and biological evaluation of dibenz[*b*,*f*][1,5]oxazocine derivatives for agonist activity at κ-opioid receptor

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ABSTRACT

A short and high yield synthetic route to dibenz[*b*,*f*][1,5]oxazocines has been developed using Pd catalyzed intramolecular cycloamination reaction. Receptor binding assay using [^{125}I]-dynorphin demonstrated that one of the derivative, **5b** showed selective κ-opioidergic property.

1. Introduction

We have been engaged in establishing new synthetic methods to benzo fused medium ring heterocycles [1,2] for quite sometimes, we noted that dibenz[*b*,*f*][1,5]oxazocines are effective as neurotropic agents, e.g. antidepressants, analgesics, and sedatives [3–5]. These are structurally similar to azocine derivatives, some of which have been shown to possess CNS activities [6,7]. The benzoxazocine nefopam is frequently used as a non-opioid analgesic and its analgesic effect on acetic acid-induced visceral nociception has been shown to be antagonized by the opioid receptor antagonist naloxone or naltrindole [8]. Among other azocines, ethyl keto cyclazocine, bremazocine, pentazocine etc. have been widely used as κ-opioid receptor (KOR) agonists. Although various applications of dibenzo fused medium ring heterocycles are reported in literature [9,10], their use as KOR agonist is not explored. We therefore undertook a project on the synthesis of some dibenzoxazocine derivatives and testing the compounds for activity at KOR.

There have been several approaches to the synthesis of dibenzo fused seven- and eight-membered ring compounds using ring-closing metathesis, palladium-induced intramolecular cyclization, or other methods have been reported in the literature [11]. However, applications of these methodologies to the preparation of dibenzoxazocine ring are scarce. A few methods for the synthesis of this useful system have been reported as in References [12,13]. Thus, the development of a suitable procedure for the synthesis of this ring system remains a valid preoccupation for synthetic organic chemists. Over the past few years' intramolecular C–N ring closure has been shown to offer a better alternative to the synthesis of medium ring heterocycles. As a part of the ongoing program in our laboratory on benzo and dibenzo fused medium ring compounds [14], we have developed various Pd catalyzed C–N/O bond forming cyclization strategies based on intramolecular Buchwald-Hartwig aryl amination [15,16] or aryl etherification reactions [17]. To the best of our knowledge, only one example of preparing dibenzoxazocines by aryl amination reaction was reported as in Reference [18].

In continuation of our efforts [15,16], we studied the applicability of such carbon-nitrogen bond forming reactions during the final cyclization step.

2. Results and discussions

2.1. Chemistry

The starting materials **3a**–**d** were prepared [14] in 92–95% yield by benzylation of commercially available substituted

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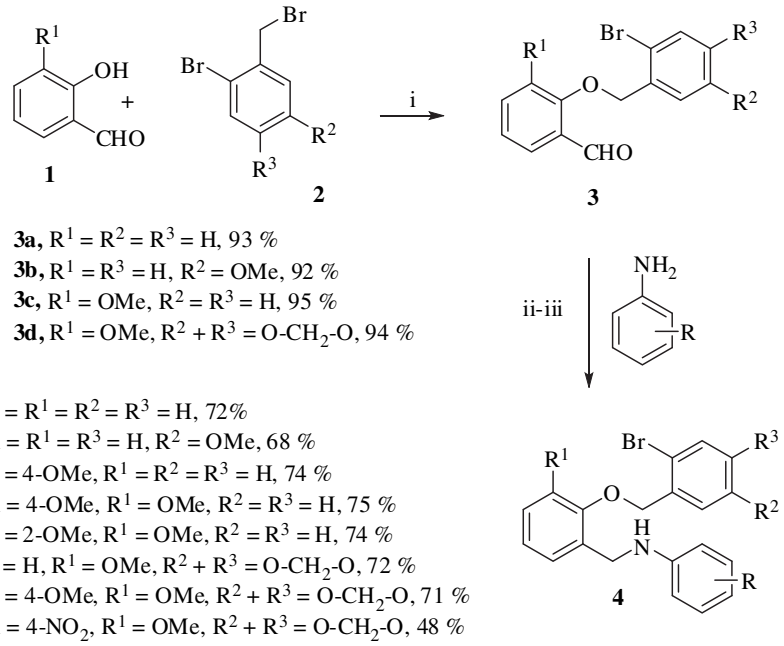


Scheme 1. Synthesis of *O*-bromobenzylated aromatic amines. Reagents and conditions: (i) acetone, K_2CO_3, reflux, 3 h (ii) EtOH, 25 °C, 12 h (iii) $NaBH_4$, EtOH, 0 °C, 2 h.

salicylaldehydes with substituted 2-bromo benzyl bromides in the presence of anhydrous potassium carbonate (Scheme 1). Imine formation with substituted anilines and subsequent $NaBH_4$ reduction in ethanol afforded the desired amines **4a**–**g** in good yield (Scheme 1). The spectral data of **4a**–**g** are in excellent agreement with the assigned structures. The lower yield obtained with 4-nitro aniline, which could not be significantly improved even by increasing temperature of the reaction, may be attributed to the electron withdrawing effect of the nitro group.

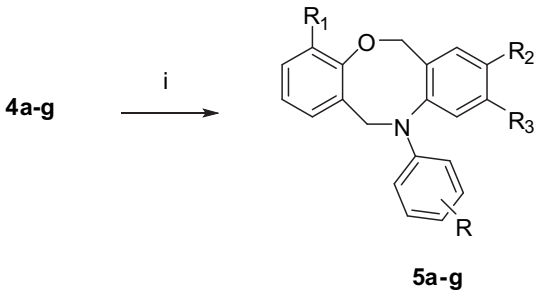
Initially we attempted to synthesize the desired cyclic product **5a** through Pd catalyzed intramolecular cycloamination reaction on **4a** pursuing the conditions reported by Buchwald et al. [19]. Entry 1 of Table 1 indicates that under these conditions the desired cyclic compound was not obtained. More recently, this aryl amination chemistry has undergone optimization, particularly with the development of new ligand systems, and the intramolecular version has been utilized for the synthesis of heterocyclic compounds [20]. We, therefore, tried to optimize the reaction conditions with different ligands, catalysts, solvents, and bases (Table 1).

Table 1
Optimization of the cycloamination reaction.

Entry	Substrate	Catalyst	Ligand	Base	Solvent	Product	Yield (%)
1	**4a**	$Pd(PPh_3)_4$	–	K_2CO_3	Toluene	**5a**	–[a]
2	**4a**	$Pd_2(dba)_3$	±BINAP	K_2CO_3+KO-*t*Bu	Toluene	**5a**	72[b]
3	**4a**	$Pd_2(dba)_3$	±BINAP	KO-*t*Bu	Toluene	**5a**	30
4	**4a**	$Pd(OAc)_2$	±BINAP	KO-*t*Bu	Toluene	**5a**	20
5	**4a**	$Pd_2(dba)_3$	PPh_3	K_2CO_3+KO-*t*Bu	Toluene	**5a**	15
6	**4a**	$Pd_2(dba)_3$	±BINAP	Cs_2CO_3	Toluene	**5a**	25
7	**4b**	$Pd_2(dba)_3$	±BINAP	K_2CO_3+KO-*t*Bu	Toluene	**5b**	77[b]
8	**4b**	$Pd_2(dba)_3$	±BINAP	K_2CO_3+KO-*t*Bu	EtOH	**5b**	–[a]
9	**4b**	$Pd_2(dba)_3$	±BINAP	K_2CO_3+KO-*t*Bu	THF	**5b**	–[a]
10	**4b**	$Pd_2(dba)_3$	±BINAP	K_2CO_3+KO-*t*Bu	Dioxane	**5b**	35
11	**4b**	$Pd_2(dba)_3$	DPPF	K_2CO_3+KO-*t*Bu	Toluene	**5b**	24

[a] No desired product was found.
[b] Optimized reaction condition.

Among the various catalysts used in the synthesis of dibenzoxazocines, $Pd_2(dba)_3$ gave the best result (Table 1, entry 2 and 7). Use of a proper base was also crucial to obtain good yield; we got only 30% product by using KO-*t*Bu, (Entry 3, Table 1), but the combined effect of KO-*t*Bu and K_2CO_3 produced significant increase in the yield. Cs_2CO_3 was found to be less effective in this reaction (Entry 6, Table 1). A study of the solvent effect (THF, EtOH, toluene, 1,4-dioxane) suggested that toluene was the solvent of choice. Application of the reagents $Pd_2(dba)_3$, ±BINAP, and KO-*t*Bu + K_2CO_3 in toluene (Scheme 2) gave the desired product **5b** in 77% yield.



Scheme 2. Synthesis of dibenzoxazocines by intramolecular cycloamination reaction. Reagents and conditions: (i) 10 mol% $Pd_2(dba)_3$, 7 mol % ±BINAP, 2.0 equiv K_2CO_3, 2.0 equiv KO-*t*Bu, dry toluene, reflux, 16–18 h.

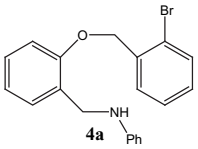
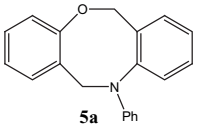
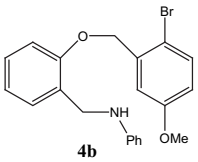
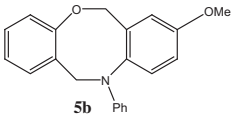
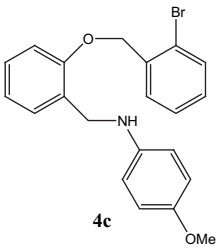
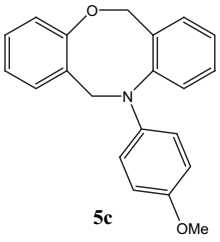
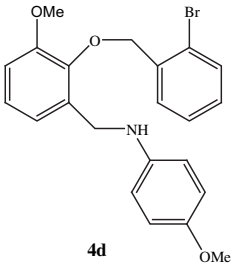
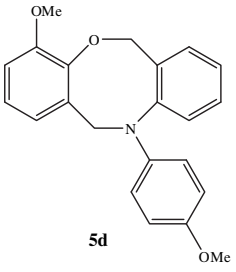
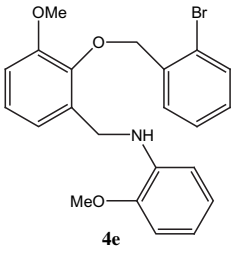
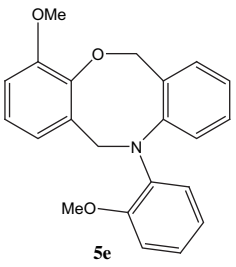
After achieving the optimized results, other substrates **4b**–**h** were similarly treated under optimized reaction conditions to afford the corresponding cyclized products **5b**–**g** (Table 2), though no cyclized product was formed with **4h**. The basicity of the amine seems to be very important for the Pd catalyzed aryl amine cyclization. In the presence of nitro group, probably it was difficult to form amine coordination complex in the catalytic cycle [21,22].

The structures of the compounds **5** were determined on the basis of spectroscopic studies supported by a single crystal X-ray analysis [23–25] on **5b** (Fig. 1).

2.2. Binding studies

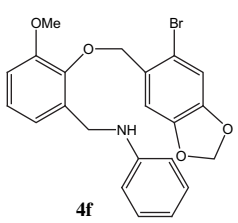
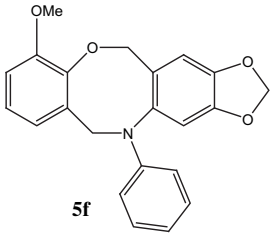
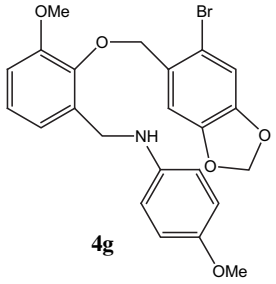
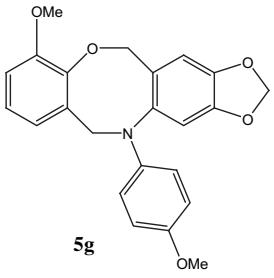
The effect of the dibenz[*b*,*f*][1,5]oxazocines derivatives on [^{125}I]-dynorphin and [^{125}I] DAMGO binding to membranes, prepared

Table 2
Reaction times and yields of the cycloamination reactions.

Entry	Precursor	Product	Time (h)	Yield[a] (%)
1	 **4a**	 **5a**	18	72
2	 **4b**	 **5b**	17	77
3	 **4c**	 **5c**	16	75
4	 **4d**	 **5d**	15	70
5	 **4e**	 **5e**	16	68

(*continued on next page*)

Table 2 (*continued*).

Entry	Precursor	Product	Time (h)	Yield[a] (%)
6	 **4f**	 **5f**	16	75
7	 **4g**	 **5g**	17	65

[a] Isolated yield.

from mouse brain, were determined. While the KOR specific ligand U50488H, at 5 μM, inhibited specific [^{125}I]-dynorphin binding by approximately 49%, only **5b** showed significant interaction at KOR binding site at similar concentrations (Table 3). However, none of the compounds were found to displace the specific [^{125}I] DAMGO binding significantly.

Affinity studies for the interaction of **5b** at the KOR were done by calculating the IC_{50} values (Fig. 2). It was observed that **5b** had high affinity for KOR with an IC_{50} of 2.17 μM, which was comparable to that of the KOR selective ligand U50488H (IC_{50} of 1.99 μM) as reported by us earlier [26].

KOR agonists and not antagonists cause transient activation of pERK [27] in cultured cells within a few minutes of application. Receptor specific antagonists inhibited such stimulatory effect on pERK by the opioid agonists. The above observations were applied in cultures of C_6 glioma with a view to determining the κ-opioid agonistic or antagonistic property of the test compounds. Cells were treated with the test compounds for 10 min and pERK levels were estimated by western blotting analysis (Fig. 3). Like the κ-opioid ligand U50488H, **5b** also stimulated ERK phosphorylation. However, in presence of the KOR antagonist norBNI, **5b** failed to evoke ERK phosphorylation, suggesting the mediation of KOR in the action of **5b**. Of the other compounds tested, none stimulated ERK phosphorylation to a significant extent (see Supplementary data online).

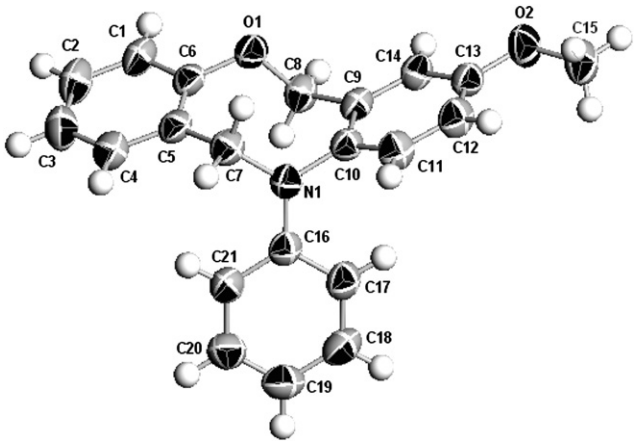


Fig. 1. ORTEP diagram of compound **5b**.

Table 3
Effect of synthetic compounds on the specific bindings[a] of κ and μ opioid receptors.

Compound	Specific binding (fmol/mg protein)	
	κ	μ
–	20.80 ± 0.48 (100)	11.04 ± 0.39 (100)
U50488H	10.40 ± 1.30* (50)	–
Naloxone	–	6.66 ± 0.38* (60)
5a	18.10 ± 1.10 (87)	8.89 ± 0.48 (80)
5b	6.14 ± 0.41* (29)	10.94 ± 0.74 (99)
5c	16.94 ± 0.50 (81)	9.54 ± 0.51 (86)
5d	17.01 ± 1.55 (81)	10.40 ± 0.32 (90)
5e	24.80 ± 0.37 (119)	9.9 ± 0.52 (89)
5f	21.20 ± 0.54 (101)	8.69 ± 1.02 (78)
5g	21.20 ± 1.98 (101)	15.29 ± 0.78* (138)

*$p < 0.05$ vs untreated control (Total binding) using one way ANOVA. Values within parenthesis represent percentage binding compared to total binding in absence of any test compound, which is taken as 100.

[a] Bindings at κ and μ opioid receptors were investigated using [^{125}I]-dynorphin and [^{125}I] DAMGO respectively, in the absence and presence of test compounds. The test compounds were used at the concentration of 5 μM for studying the displacement of both [^{125}I]-dynorphin and [^{125}I] DAMGO. Binding at κ and μ opioid receptors was determined from the difference in binding in absence and presence of 5 μM of both U50488H (κ) and naloxone (μ) respectively. The binding was carried out in mouse brain membrane. Data represent Mean ± SEM of at least three experiments.

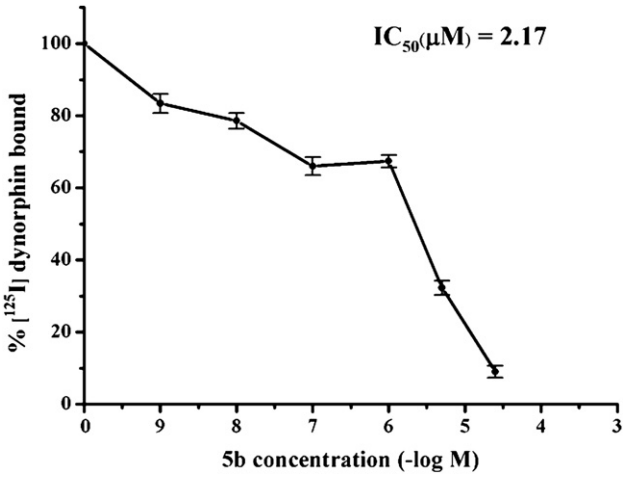


Fig. 2. Competitive inhibition specific [^{125}I]-dynorphin binding to brain membrane by **5b**. Each point represents mean of three individual determinations. IC_{50} concentrations were determined.

It is common that opioids exert their actions through three types of receptors, μ, κ and δ, but the relationships between opioid receptors and physical and psychic dependences vary with the receptor subtype [28]. Although μ opioid receptor (MOR) mediates most of the opioid actions including analgesia, tolerance and reward [29,30], increasing evidence indicates that the activation of the κ-receptor opposes a variety of μ-receptor mediated actions [31] like analgesia [32], tolerance [33] and withdrawal [34]. Therefore, the use of κ-opioid agonists [35,36] is an important approach towards exploring potential treatments of stimulant abuse. However, KOR agonists produce side effects including sedation and vomiting [35], which has led to the search for other KOR agonists of varied structures like the azocines (cyclazocine, bremazocine, pentazocine etc.), arylacetamides (U50488H, U69593, R84760 etc.), epoxymorphinan nalfurafine, ibogaine congeners, salvinorin A etc.

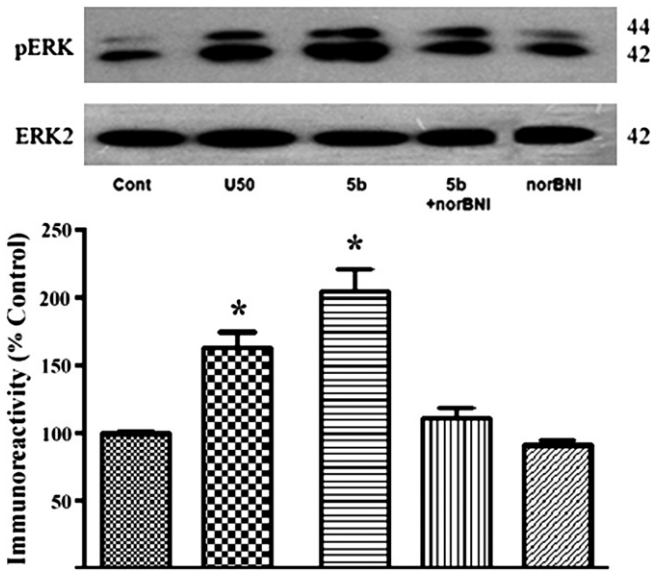


Fig. 3. Effect of test compound **5b** on the induction of pERK activity in C6 glioma cells. Five-day old cultures were serum starved for 24 h. The effects of k-opioid receptor agonist U50488H (1 μM) and the test compound **5b** (40 μM) were evaluated by treating cells for 10 min with the drugs and quantitating pERK1/2 by western blot analysis. Stimulation of pERK by **5b** was antagonized by co-treatment with norBNI (1 μM). The relative intensities of the pERK bands, indicated in the figure, were obtained by densitometric scanning by ImageJ. Results are mean ± SEM of at least three blots. *$p < 0.01$ versus untreated control.

3. Conclusions

In conclusion, we have established a straightforward three-step synthetic route to dibenzo fused eight-membered oxazacycles using palladium catalyzed intramolecular cycloamination reaction as the key step. The present study identifies a novel dibenzoxazocine derivative having κ-opioid agonist activity. Development of safe κ-agonists has become relevant in recent times as a replacement of morphine and dibenzoxazocines afford an interesting structure for further investigation in this area. We also plan to use the test compound for attenuating physical dependence in animals exposed chronically to drugs of abuse.

4. Experimental section

4.1. Chemistry

4.1.1. General

Some reagents were obtained from commercial sources and used without purification. The solvents used were of technical grade, and freshly distilled prior to use. All melting points were obtained on a laboratory devices melting point bath and are uncorrected. ^{1}H (300 MHz, 600 MHz) and ^{13}C (75 MHz, 150 MHz) NMR spectra were recorded using $CDCl_3$ and DMSO-d_6 as solvent and tetramethyl silane (TMS) as internal standard on Bruker DPX 300 MHz and Bruker DRX 600 MHz NMR instruments at ambient temperature. Chemical shifts are stated in parts per million in δ scales. Infrared spectra were recorded on a JASCO-FTIR Model-410, using KBr pellets. Mass spectra were measured in ESIMS (+) and HRMS mode. ESIMS were done on a Waters Micromass Q-TOF micro™ Mass Spectrometer. X-Ray crystallographic data of single crystals were collected on Bruker Kappa Apex II with Mo-Kα radiation (λ = 0.71073 Å). TLC was performed on pre-coated plates (0.25 nm, silica gel 60 F_{254}).

4.1.2. *See Reference [3] for the procedure of the compounds **3a–d** and for spectral data of the compounds **3a–c***

*4.1.2.1. 2-(6-Bromo-benzo[1,3]dioxol-5-ylmethoxy)-3-methoxy-benzaldehyde (**3d**).* White crystalline solid, mp 153–156 °C, Yield 94%. IR (KBr, ν_{max}) 3050, 2920, 2889, 1687,1587, 1482, 1380, 1315, 1250, 1224, 1176, 1107, 1037, 1008, 930, 871, 768, 673 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.95 (s, 3H), 5.18 (s, 2H), 5.99 (s, 2H), 7.01 (d, J = 12.0 Hz, 2H), 7.15–7.17 (m, 2H), 7.39–7.41 (m, 1H), 10.31 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.1 (CH_3), 75.2 (CH_2), 101.9 (CH_2), 110.3 (CH), 112.8 (CH), 114.5 (C), 118.0 (CH), 119.0 (CH), 124.4 (CH), 129.1 (C), 130.2 (C), 147.5 (C), 148.4 (C), 150.8 (C), 152.9 (C), 190.3 (CH). MS (ESI$^+$): m/z = 385, 387 (M + Na$^+$ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{16}H_{13}BrO_5$: C 56.22, H 4.16; Found C 56.40, H 4.04.

4.1.3. General procedure for the synthesis of O-bromobenzylated aromatic amines **4a–h**

To an ethanolic solution of **3**, substituted anilines were added and the reaction mixture was stirred at room temperature for 12 h. After cooling to 0 °C, sodium borohydride was added portionwise and the reaction mixture was stirred for 2 h (TLC). The solvent was removed via rotary evaporation. The reaction mixture was extracted with CH_2Cl_2 (2 × 10 ml), and then washed with saturated sodium bicarbonate solution and water (2 × 8 ml). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The crude residue was purified via flash column

chromatography over silica gel (230–400 mesh) eluting with 2–4% ethyl acetate in pet. ether to get the compounds **4a**–**h**.

*4.1.3.1. [2-(2-Bromo-benzyloxy)-benzyl]-phenyl-amine (**4a**).* White solid, mp 64–66 °C, Yield 72%; IR (KBr, ν_{max}): 3399, 2924, 1602, 1500, 1450, 1362, 1245, 1092, 1044, 1026, 750, 688 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 4.27 (d, $J = 5.4$ Hz, 2H), 5.13 (s, 2H), 6.06 (br. s, 1H), 6.52 (d, $J = 7.2$ Hz, 2H), 6.91 (t, $J = 7.2$ Hz, 1H), 6.99–7.09 (m, 3H), 7.19–7.34 (m, 4H), 7.41 (d, $J = 6.3$ Hz, 1H), 7.67 (dd, $J = 7.5$, 18.3 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 43.7 (CH_2), 69.4 (CH_2), 111.6 (CH), 113.1 (2 × CH), 117.4 (CH), 121.1 (CH), 122.3 (C), 127.6 (CH), 128.3 (CH), 128.8 (CH), 129.1 (CH), 129.2 (2 × CH), 129.3 (CH), 132.6 (CH), 136.2 (C), 148.2 (C), 156.1 (C). MS (ESI^+): $m/z = 390$, 392 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{20}H_{18}BrNO$: C 65.23, H 4.93, N 3.80; Found C 65.50, H 5.12, N 4.02.

*4.1.3.2. [2-(2-Bromo-5-methoxy-benzyloxy)-benzyl]-phenyl-amine (**4b**).* White solid, mp 104–107 °C, Yield 68%; IR (KBr, ν_{max}): 3413, 2999, 2934, 2841, 1600, 1481, 1456, 1365, 1277, 1243, 1157, 1055, 1014, 867, 798, 749 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 3.71 (s, 3H), 4.28 (d, $J = 5.4$ Hz, 2H), 5.15 (s, 2H), 6.03 (br. s, 1H), 6.48–6.55 (m, 3H), 6.90 (d, $J = 7.5$ Hz, 2H), 6.99–7.09 (m, 3H), 7.22–7.29 (m, 3H), 7.58 (d, $J = 8.7$ Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 43.7 (CH_2), 55.2 (CH_3), 69.2 (CH_2), 111.6 (CH), 112.1 (C), 112.9 (2 × CH), 113.8 (CH), 115.1 (CH), 117.3 (CH), 121.1 (CH), 127.5 (C), 128.4 (CH), 129.1 (2 × CH), 129.2 (CH), 133.1 (CH), 137.1 (C), 148.3 (C), 156.0 (C), 159.1 (C). MS (ESI^+): $m/z = 420$, 422 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{21}H_{20}BrNO_2$: C 63.33, H 5.06, N 3.52; Found C 63.05, H 4.87, N 3.77.

*4.1.3.3. [2-(2-Bromo-benzyloxy)-benzyl]-(4-methoxy-phenyl)-amine (**4c**).* White solid, mp 105-107 °C, Yield 74%; IR (KBr, ν_{max}): 3386, 2952, 2899, 2846, 1597, 1513, 1455, 1372, 1293, 1245, 1122, 1032, 818, 756 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6): δ 3.60 (s, 3H), 4.23 (d, $J = 5.7$ Hz, 2H), 5.19 (s, 2H), 5.63 (br. s, 1H), 6.48 (d, $J = 8.7$ Hz, 2H), 6.66 (d, $J = 8.7$ Hz, 2H), 6.90 (t, $J = 7.2$ Hz, 1H), 7.07 (d, $J = 8.1$ Hz, 1H), 7.19–7.34 (m, 3H), 7.43 (t, $J = 7.0$ Hz, 1H), 7.66 (dd-like, $J = 7.2$, 20.7 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 44.7 (CH_2), 55.8 (CH_3), 69.4 (CH_2), 111.6 (CH), 114.5 (2 × CH), 114.8 (2 × CH), 121.1 (CH), 122.4 (C), 127.7 (CH), 128.0 (C), 128.3 (CH), 128.8 (CH), 129.2 (CH), 129.3 (CH), 132.7 (CH), 136.2 (C), 142.6 (C), 152.1 (C), 156.2 (C). MS (ESI^+): $m/z = 420$, 422 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{21}H_{20}BrNO_2$: C 63.33, H 5.06, N 3.52; Found C 63.07, H 4.81, N 3.27.

*4.1.3.4. [2-(2-Bromo-benzyloxy)-3-methoxy-benzyl]-(4-methoxy-phenyl)-amine (**4d**).* White solid, mp 75–78 °C, Yield 75%; IR (KBr, ν_{max}): 3422, 2932, 1585, 1516, 1473, 1436, 1376, 1305, 1261, 1224, 1183, 1084, 1058, 982, 952, 820, 788, 759 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6): δ 3.60 (s, 3H), 3.84 (s, 3H), 4.09 (d, $J = 5.7$ Hz, 2H), 5.10 (s, 2H), 5.58 (br. s, 1H), 6.38 (d, $J = 8.7$ Hz, 2H), 6.63 (d, $J = 8.7$ Hz, 2H), 6.86 (d, $J = 6.6$ Hz, 1H), 6.95–7.04 (m, 2H), 7.30 (t, $J = 7.2$ Hz, 1H), 7.41 (t, $J = 7.2$ Hz, 1H), 7.58 (d, $J = 7.2$ Hz, 1H), 7.66 (d, $J = 7.8$ Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 44.3 (CH_2), 55.8 (2 × CH_3), 73.8 (CH_2), 111.5 (CH), 114.3 (2 × CH), 114.8 (2 × CH), 120.9 (CH), 122.9 (C), 124.3 (CH), 127.5 (CH), 129.2 (CH), 129.7 (CH), 132.5 (CH), 133.7 (C), 137.2 (C), 142.4 (C), 145.6 (C), 152.0 (C), 152.7 (C). MS (ESI^+): $m/z = 450$, 452 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{22}H_{22}BrNO_3$: C 61.69, H 5.18, N 3.27; Found C 61.48, H 4.95, N 3.01.

*4.1.3.5. [2-(2-Bromo-benzyloxy)-3-methoxy-benzyl]-(2-methoxy-phenyl)-amine (**4e**).* White solid, mp 108–111 °C, Yield 74%; IR (KBr, ν_{max}): 3427, 3016, 2930, 2838, 1599, 1518, 1477, 1459, 1351, 1274, 1216, 1092, 1056, 1022, 898, 777, 735 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 3.74 (s, 3H), 3.84 (s, 3H), 4.20 (d, $J = 6.0$ Hz, 2H), 5.10 (s, 2H), 5.27 (br. s, 1H), 6.22 (d, $J = 7.5$ Hz, 1H), 6.49 (t-like, $J = 7.2$ Hz, 1H), 6.62 (t-like, 1H), 6.76 (d, $J = 7.8$ Hz, 1H), 6.81 (d, $J = 6.0$ Hz, 1H), 6.98–7.03 (m, 2H), 7.30 (t-like,1H), 7.42 (t, $J = 7.3$ Hz, 1H), 7.58 (d, $J = 7.2$ Hz, 1H), 7.66 (d, $J = 7.8$ Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 42.8 (CH_2), 55.4 (CH_3), 55.9 (CH_3), 73.7 (CH_2), 109.3 (CH), 110.1 (CH), 111.4 (CH), 116.3 (CH), 120.7 (CH), 121.2 (CH), 122.6 (C), 124.3 (CH), 127.4 (CH), 129.1 (CH), 129.5 (CH), 132.4 (CH), 133.6 (C), 137.4 (C), 138.1 (C), 145.6 (C), 146.8 (C), 152.7 (C). MS (ESI^+): $m/z = 450$, 452 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{22}H_{22}BrNO_3$: C 61.69, H 5.18, N 3.27; Found C 61.44, H 4.92, N 3.48.

*4.1.3.6. [2-(6-Bromo-benzo[1,3]dioxol-5-ylmethoxy)-3-methoxy-benzyl]-phenyl-amine (**4f**).* White solid, mp 95–98 °C, Yield.72%; IR (KBr, ν_{max}): 3398, 1604, 1502, 1479, 1272, 1251, 1204, 1038, 1010, 826, 751, 692 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6): δ 3.85 (s, 3H), 4.12 (d-like, $J = 5.7$ Hz, 2H), 5.02 (s, 2H), 6.08 (s, 2H), 6.41–6.50 (m, 3H), 6.83–6.88 (m, 1H), 6.98–7.02 (m, 4H), 7.08 (s, 1H), 7.25 (s, 1H). MS (ESI^+): $m/z = 464$, 466 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{22}H_{20}BrNO_4$: C 59.74, H 4.56, N 3.17; Found C 60.02, H 4.81, N 2.93.

*4.1.3.7. [2-(6-Bromo-benzo[1,3]dioxol-5-ylmethoxy)-3-methoxy-benzyl]-(4-methoxy-phenyl)-amine (**4g**).* White solid, mp 120–123 °C, Yield.71%; IR (KBr, ν_{max}): 3400, 1610, 1506, 1490, 1270, 1265, 1190, 1030, 1012, 835, 760 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6): δ 3.60 (s, 3H), 3.84 (s, 3H), 4.07 (br. s, 2H), 5.00 (s, 2H), 5.58 (br. s, 1H), 6.07 (s, 2H), 6.38 (d-like, $J = 8.1$ Hz, 2H), 6.63 (d-like, $J = 7.0$ Hz, 2H), 6.85 (br. s, 1H), 6.96 (br. s, 1H), 7.07 (s, 1H), 7.25 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 44.3 (CH_2), 55.8 (CH_3), 55.9 (CH_3), 73.8 (CH_2), 101.8 (CH_2), 110.0 (CH), 111.5 (CH), 112.6 (CH), 113.8 (C), 114.2 (2 × CH), 114.8 (2 × CH), 120.9 (CH), 124.3 (CH), 130.4 (C), 133.7 (C), 142.4 (C), 145.5 (C), 147.4 (C), 148.0 (C), 152.1 (C), 152.6 (C). MS (ESI^+): $m/z = 472$, 474 (M + H^+ for ^{79}Br, ^{81}Br) and 494, 496 (M + Na^+ for ^{79}Br, ^{81}Br). Anal. calcd. for $C_{23}H_{22}BrNO_5$: C 58.49, H 4.69, N 2.97; Found C 58.28, H 4.41, N 3.18.

4.1.4. General procedure for the cycloamination reaction

To a stirred solution of *O*-bromobenzylated amine (1 mmol) in dry toluene (15 mL/mmol) were added KO-*t*Bu (135 mg, 2 equiv), anhydrous K_2CO_3 (167 mg, 2 equiv), $Pd_2(dba)_3$ (10 mol %), and ±BINAP (7 mol %), and the reaction mixture was refluxed for the stipulated time (Table 2) under nitrogen atmosphere. After completion of the reaction (monitored by TLC), toluene was evaporated under vacuum, and the crude reaction mixture was extracted with ethyl acetate (3 × 25 ml). The organic layer was washed with water (3 × 25 ml) followed by brine (2 × 15 ml). The total organic layer was dried over sodium sulfate, filtered, and the solvent was evaporated under reduced pressure. The crude mass was purified by flash column chromatography to get the desired compound.

*4.1.4.1. 11- Phenyl-11,12-dihydro-6H-dibenz[b,f][1,5]oxazocine (**5a**).* Yellow solid; mp 70 °C, Yield: 72%; IR (KBr, ν_{max}): 2924, 1596, 1495, 1448, 1371, 1265, 1213, 1104, 973, 766, 744 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ = 4.76 (s, 2H), 4.94 (s, 2H), 6.63–6.68 (m, 3H), 7.02–7.20 (m, 5H), 7.25–7.32 (m, 2H), 7.37–7.45 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 53.3, 76.0, 113.6, 117.2, 122.4, 123.5, 126.4, 128.8, 129.2, 130.1, 130.6, 131.0, 131.6, 136.6, 147.2, 147.6, 160.7. MS (ESI^+): $m/z = 310$ $(M + Na)^+$. HRMS: 310.1198 $(M + Na)^+$ [expected 310.1208 $(M + Na)^+$].

*4.1.4.2. 2-Methoxy-11- phenyl-11,12-dihydro-6H-dibenz[b,f][1,5]oxazocine (**5b**).* Pale yellow solid, mp 85 °C, Yield 77%; IR (KBr, ν_{max}): 2922, 1724, 1599, 1498, 1372, 1269, 1039, 982 cm^{-1} ^1H NMR ($CDCl_3$, 300 MHz): δ 3.84 (s, 3H), 4.71 (s, 2H), 4.84 (s, 2H), 6.57–6.65 (m, 3H), 6.91–6.97 (m, 2H), 7.02–7.12 (m, 4H), 7.15–7.24 (m, 2H), 7.42 (d, $J = 7.2$ Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 53.8, 55.5, 76.1, 112.9, 115.2, 115.9, 116.7, 122.6, 123.7, 128.8, 130.6, 131.3, 131.6, 138.3, 140.4,

147.6, 158.0, 160.8. EI MS (ESI^+): m/z 340 $(M + Na)^+$. HRMS: 340.1314 $(M + Na)^+$ [expected 340.1313 $(M + Na)^+$].

*4.1.4.3. 11-[4-Methoxy-phenyl]-11,12-dihydro-6H-dibenz[b,f][1,5] oxazocine (**5c**).* Yellow solid, mp 84 °C, Yield: 75%; IR (KBr, ν_{max}): 2920, 2853, 1726, 1600, 1495, 1370, 1271, 1042, 979, 858, 746 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ = 3.70 (s, 3H), 4.76 (s, 2H), 5.01 (s, 2H), 6.68 (q, J = 9.0 Hz, 4H), 7.02–7.03 (m, 2H), 7.14–7.20 (m, 2H), 7.24 (d, J = 3.4 Hz, 1H), 7.30–7.40 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 53.5, 55.6, 75.7, 114.5, 116.2, 121.9, 123.3, 125.1, 128.2, 128.8, 129.8, 130.1, 131.0, 131.4, 135.2, 141.4, 148.3, 152.2, 160.2. MS (ESI^+): m/z = 318 $(M + H)^+$. Anal. Calcd for $C_{21}H_{19}NO_2$: C, 79.47; H, 6.03; N, 4.41. Found: C, 79.29; H, 6.21; N, 4.66.

*4.1.4.4. 10-Methoxy-11-[4-methoxy-phenyl]-11,12-dihydro-6H-dibenz[b,f][1,5]oxazocine (**5d**).* White solid, mp 88 °C, Yield: 70%; IR (KBr, ν_{max}): 2924, 2840, 1722, 1598, 1550, 1460, 1360, 1240, 1170, 1130, 1080, 990, 812, 699 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ = 3.70 (s, 3H), 3.84 (s, 3H), 4.73 (s, 2H), 5.0 (s, 2H), 6.66 (dd, J = 8.4 Hz, 3H), 6.80–6.82 (m, 2H), 6.95–6.97 (m, 2H), 7.17–7.25 (m, 2H), 7.31–7.41 (m, 2H). MS (ESI^+): m/z = 370 $(M + Na)^+$. Anal. Calcd for $C_{22}H_{21}NO_3$: C, 76.06; H, 6.09; N, 4.03. Found: C, 75.87; H, 5.89; N, 4.25.

*4.1.4.5. 10-Methoxy-11-[2-methoxy-phenyl]-11,12-dihydro-6H-dibenz[b,f][1,5]oxazocine (**5e**).* White solid, mp 89 °C, Yield: 68%; IR (KBr, ν_{max}): 2931, 2835, 1597, 1576, 1496, 1454, 1361, 1346, 1262, 1212, 1183, 1122, 1078, 998, 745 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ = 3.77 (s, 3H), 3.84 (s, 3H), 4.90 (s, 2H), 5.36 (s, 2H), 6.68–6.98 (m, 7H), 7.06–7.08 (m, 2H), 7.16–7.25 (m, 1H), 7.36–7.40 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 53.6, 55.6, 56.1, 75.5, 111.5, 112.4, 121.3, 122.2, 123.0, 123.8, 124.4, 127.0, 128.5, 129.0, 130.0, 130.2, 131.1, 136.9, 148.3, 150.4, 151.1, 154.4. MS (ESI^+): m/z = 370 $(M + Na)^+$. Anal. Calcd for $C_{22}H_{21}NO_3$: C, 76.06; H, 6.09; N, 4.03. Found: C, 75.92; H, 6.20; N, 3.88.

*4.1.4.6. 2,3-(Methylenedioxy)-10-Methoxy-11-phenyl-11,12-dihydro-6H-dibenz[b,f][1,5]oxazocine (**5f**).* White solid, mp 98 °C, Yield: 75%; IR (KBr, ν_{max}): 2936, 2840, 1608, 1585, 1453, 1367, 1260, 1179, 1130, 742 cm^{-1}.^1H NMR (300 MHz, $CDCl_3$): δ = 3.84 (s, 3H), 4.69 (s, 2H), 4.80 (s, 2H), 6.00 (s, 2H), 6.60–6.68 (m, 3H), 6.75–6.82 (m, 3H), 6.91 (s, 2H), 7.00–7.02 (m, 1H), 7.11 (m, 1H). MS (ESI^+): m/z = 384 $(M + Na)^+$. Anal. Calcd for $C_{22}H_{19}NO_4$: C, 73.12; H, 5.30; N, 3.88. Found: C, 73.35; H, 5.49; N, 3.64.

*4.1.4.7. 2,3-(Methylenedioxy)-10-Methoxy-11-[4-methoxy-phenyl]-11,12-dihydro-6H-dibenz[b,f][1,5]oxazocine (**5g**).* White solid, mp 102 °C, Yield: 65%; IR (KBr, ν_{max}): 2933, 2850, 1604, 1597, 1470, 1380, 1302, 1145, 745 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ = 3.70 (s, 3H), 3.83 (s, 3H), 4.66 (s, 2H), 4.82 (s, 2H), 5.98 (s, 2H), 6.58 (s, 2H), 6.72–6.97 (m, 7H). MS (ESI^+): m/z = 414 $(M + Na)^+$. Anal. Calcd for $C_{23}H_{21}NO_5$: C, 70.49; H, 5.51; N, 3.58. Found: C, 70.72; H, 5.29; N, 3.79.

4.2. Biology

4.2.1. Materials

DMEM, F12 and FBS were obtained from Gibco-BRL, Life Technologies. Trypsin, soybean trypsin inhibitor, U50488H, Naloxone hydrochloride, Dynorphin, DAMGO, and anti-mouse IgG-HRP conjugated 2nd antibody were from Sigma Chemical Co. (USA). Primary antibody of pERK1/2 was purchased from Santacruz (USA). $Na^{125}I$ was from Perkin Elmer (Boston, MA). 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, adult albino Balb/c mice, 20–30 g, were used. Animals were housed four 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

C6 glioma cells were used in the study. Cells were grown in DMEM containing 10% fetal calf serum, 50 μg/ml gentamicin, penstrep in 5% CO_2 at 37 °C.

4.2.4. Membrane preparation

For opioid receptor binding studies, membrane was prepared from mouse brain. The mouse was sacrificed, brain was dissected out, homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4), and centrifuged at 20,000 g for 30 min. The pellet was resuspended in the same buffer, incubated for 20 min at 37 °C, and centrifuged as above. The pellet was resuspended in ice-cold buffer and used for binding assay. Protein concentration was determined by the method described by Lowry et al. [37] (1951) using bovine serum albumin (BSA) as standard.

4.2.5. Preparation of ^{125}I-dynorphin

Dynorphin was labeled with [^{125}I] sodium iodide as described by Gairin et al. [38]. The reaction was initiated by adding 10 μl of chloramine T (2 μg/μl) dissolved in a 0.2 M phosphate buffer (pH 7.4) to 10 μl of dynorphin (1 μg/μl) dissolved in 0.2 M phosphate buffer and 1 mCi of $Na^{125}I$. After 40 s of reaction, the reaction was terminated by adding 40 μl of sodium metabisulfite (2 μg/μl). The reaction mixture was diluted with 0.1% aqueous trifluoroacetic acid (TFA) and absorbed in SepPak. The mixture of labeled and unlabeled dynorphin were eluted out in a 1 ml solution containing 99.9% acetonitrile with 0.1% TFA. Finally the labeled dynorphin was separated from the unlabeled dynorphin by column chromatography on a C_{18} column with elution with a mobile phase of 20:0.2:79.8 acetonitrile/TFA/water. Fractions containing the monoiodinated compound were pooled and used for subsequent studies.

4.2.6. Radioligand binding

KOR binding was carried out with mouse membrane using [^{125}I] dynorphin as performed earlier [26]. Briefly, membranes (100–200 μg of protein) were incubated with 2 nM [^{125}I] dynorphin for 30 min at 37 °C in 50 mM Tris–HCl buffer, pH 7.4. Non-specific binding was determined in the presence of 5 μM concentration of unlabeled U50488H. The different test compounds were used at the concentration of 5 μM for studying the displacement of [^{125}I]-dynorphin. The dibenzoxazocine, **5b**, which showed significant affinity for KOR was further evaluated for its IC_{50} value for displacing [^{125}I]-dynorphin using six different concentrations ranging from 1 nM to 25 μM. 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% polyethyleneimine. The filters were washed thrice with ice-cold buffer and the radioactive retained on filters were counted in a liquid scintillation counter (Wallac, model 1049–411, Perkin Elmer, USA).

4.2.7. ERK assay

ERK phosphorylation was measured by immunoblotting as described in Reference [39]. Following starvation for 24 h, cells were treated as indicated. Antagonists were added to the medium

30 min before stimulation with agonist and compounds. After the indicated stimulation period, medium was removed, and wells of the flasks 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 mM sodium vanadate, 1% Nonidet P-40, 1 mM PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin, pH 7.5). Cell lysates were centrifuged at 14,000 *g* 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 membrane 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 probed with monoclonal antibody against pERK (1:1000 dilution) for 2–3 h, followed by treating with a peroxidase conjugated secondary antibody.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.02.024.

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