Short Communication

Effect of \(\kappa\)-opioid receptor agonist on the growth of non-small cell lung cancer (NSCLC) cells

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**BACKGROUND:** It is becoming increasingly recognised that opioids are responsible for tumour growth. However, the effects of opioids on tumour growth have been controversial.

**METHODS:** The effects of \(\kappa\)-opioid receptor (KOR) agonist on the growth of non-small cell lung cancer (NSCLC) cells were assessed by a cell proliferation assay. Western blotting was performed to ascertain the mechanism by which treatment with KOR agonist suppresses tumour growth.

**RESULTS:** Addition of the selective KOR agonist U50,488H to gefitinib-sensitive (HCC827) and gefitinib-resistant (H1975) NSCLC cells produced a concentration-dependent decrease in their growth. These effects were abolished by co-treatment with the selective KOR antagonist nor-BNI. Furthermore, the growth-inhibitory effect of gefitinib in HCC827 cells was further enhanced by co-treatment with U50,488H. With regard to the inhibition of tumour growth, the addition of U50,488H to H1975 cells produced a concentration-dependent decrease in phosphorylated-glycogen synthase kinase 3β (p-GSK3β).

**CONCLUSION:** The present results showed that stimulation of KOR reduces the growth of gefitinib-resistant NSCLC cells through the activation of GSK3β.

**Keywords:** \(\kappa\)-opioid receptor; non-small cell lung cancer; gefitinib

Opioids are small endogenously produced peptide molecules that are widely known for their analgesic and psychoactive properties (Ciccone et al., 1980; Zubieta et al., 2001; Moles et al., 2004). It has been shown that opioids can promote the growth of tumour cells (Lazarczyk et al., 2010). On the other hand, it has been controversially reported that opioids induce the apoptosis of immunocytes, cancer cells and neuroblastoma cells (Boehncke et al., 2010). Thus, it is becoming increasingly recognised that opioids have a role in tumour growth (Saurer et al., 2008).

Three major types of opioid receptors, \(\mu\), \(\delta\) and \(\kappa\), have been well characterised. \(\kappa\)-Opioid receptors (KORs) are widely expressed throughout the central nervous system (Chavkin et al., 1982; Dhawan et al., 1996). It has been reported that KOR is also expressed in the human adenocarcinoma breast cancer cell line MCF7 and small cell lung carcinoma (Kallergi et al., 2003). Furthermore, KOR agonist has been shown to inhibit the growth of H157 cell, which is a non-small cell lung cancer (NSCLC) cell (Maneckjee and Minna, 1990). However, little is known about the mechanism that underlies the inhibitory effect of KOR stimulation on the growth of NSCLC cells.

**MATERIALS AND METHODS**

**Cell culture**

The human NSCLC cell lines HCC827 and NCI-H1975 (H1975; both from American Type Culture Collection Co., MD, USA) were
cultured in HEPES-modified RPMI 1640 medium (Sigma-Aldrich Co., St Louis, MO, USA) with 10% fetal bovine serum (FBS; Invitrogen Life Technologies Co., Carlsbad, CA, USA) and 1% penicillin-streptomycin (Invitrogen Life Technologies Co.). Normal human lung fibroblasts (NHLF; Lonza Inc., Allendale, NJ, USA) were cultured in fibroblast basal medium with insulin, rhFGF-B, GA-1000 and FBS (all from Takara Bio Inc., Tokyo, Japan). All cells were maintained under a humidified atmosphere of 5% CO₂ at 37°C.

Reagents
The reagents used in the present study were gefitinib (Toronto Research Chemicals Inc., Canada), (±) trans 3,4-dichloro-N-(2-(1-pyrrolidinyl) cyclohexyl)-benzeneacetamide (U50,488H) methanesulfonate (Sigma Chemical Co.), nor-binaltorphimine dihydrochloride (nor-BNI; Tocris Cookson Ltd., St Louis, MO, USA), and 6-bromoindirubin-3'-oxime (BIO; WAKO Pure Chemical Industries Ltd., Osaka, Japan).

Cell viability assay
Cell viability was determined by a cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole (MTT). A 20-μl of MTT solution (5 mg ml⁻¹) was added to each well of the culture medium. After incubation for an additional 2 h, the medium was removed and 100 μl of DMSO was added to resolve the formazan crystals. Optical density was measured using a microplate reader with an absorption wavelength of 600 nm. In each experiment, three replicates were prepared for each sample. The proportion of living cells was determined based on the difference in absorbance between the samples and controls.

Immunohistochemistry
The procedure for immunohistochemistry is described in the Supplementary Methods.

RNA preparation and semiquantitative analysis by reverse transcription (RT)-PCR
The RNA preparation and RT-PCR method are described in the Supplementary Methods.

Western blotting
Sample preparation and loading for western blotting are described in the Supplementary Methods. For immunoblot detection, membranes were blocked in Tris-buffered saline (TBS) containing 1% non-fat milk (Bio-Rad Laboratories, Hercules, CA, USA) and 0.1% Tween 20 (Sigma-Aldrich Co.) for 1 h at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS (1:1000 phosphorylated-EGFR (Cell Signaling Technology Inc., Boston, MA, USA), 1:500 p-Akt (Cell Signaling Technology Inc.), 1:1000 p-GSK3β (Cell Signaling Technology Inc.), 1:2000 p-STAT3 (Cell Signaling Technology Inc.), 1:750 GSK3β (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1:5000 Akt (Cell Signaling Technology Inc.) and 1:3500 Stat3 (Cell Signaling Technology Inc.) containing 1% non-fat dried milk with 0.1% Tween 20 overnight at 4°C. The membrane was washed in TBS containing 0.05% Tween 20, and then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates Inc., Birmingham, AL, USA) diluted 1:10,000 in TBS containing 1% non-fat dried milk containing 0.1% Tween 20. The antigen–antibody peroxidase complex was finally detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) and visualised by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL, USA).

**Figure 1** Expression of κ-opioid receptors in either NHLF, HCC827 or H1975 cells. (A) Upper: Representative RT-PCR for mRNAs of κ-opioid receptors and GAPDH, an internal standard, in each cell type. Lower: The intensity of the bands was determined semiquantitatively using ImageJ (National Institute of Health, Bethesda, MD, USA). The values for κ-opioid receptor mRNA were normalised by the value for GAPDH mRNA. Data represent the mean with s.e.m. of five independent samples (**<P<0.01, ***P<0.001 vs NHLF). (B) Distribution of the κ-opioid receptor-like immunoreactivity in either NHLF (b–i), HCC827 (b–ii) or H1975 (b–iii) cells. Scale bars = 50 μm for all panels.
RESULTS

Localisation of KORs in NSCLC cells

KORs were found in gefitinib-sensitive HCC827 cells, gefitinib-resistant H1975 cells and NHLF cells, as detected by RT-PCR (Figure 1A) and immunoreactivity towards KOR antibody (Figure 1B). The expression of KOR mRNA was significantly increased in HCC827 cells (P < 0.01 vs NHLF) and H1975 cells (P < 0.001 vs NHLF) compared with NHLF (Figure 1).

Effect of KOR agonist on the growth of the EGFR exon 19 mutant NSCLC cell line HCC827

Addition of the KOR agonist U50,488H to HCC827 cells for 2 days produced a concentration-dependent decrease in tumour cell growth (Figure 2A, P < 0.001 vs non-treated group). This effect was abolished by co-treatment with the selective KOR antagonist nor-BNI (Figure 2B, ***P < 0.001 vs non-treated group, **P < 0.01 vs U50,488H-treated group). In contrast, treatment of NHLF cells with U50,488H did not affect their growth (Figure 2C). In experiments that compared the inhibition of cell growth in cells treated with gefitinib and U50,488H with gefitinib- and U50,488H-treated cells, the growth-inhibitory effects in HCC827 cells were further enhanced in a dose-dependent manner (Figure 2D, P < 0.001 vs gefitinib-treated cells).

Changes in the growth of gefitinib-resistant H1975 cells by treatment with KOR agonist

Treatment of gefitinib-resistant H1975 cells with U50,488H for 2 days produced a concentration-dependent and dramatic decrease in tumour cell growth (Figure 3A, P < 0.001 vs non-treated group). This effect was blocked by co-treatment with nor-BNI (Figure 3B, ***P < 0.001 vs non-treated group, **P < 0.01 vs U50,488H-treated group).

Effect of KOR agonist on the levels of phosphorylated Akt, GSK3β and Stat3 in H1975 cells

There were no changes in the levels of either p-Akt or p-Stat3 in H1975 cells by treatment with U50,488H for 2 days (Figures 3C and E). However, the addition of U50,488H to H1975 cells produced a significant and concentration-dependent decrease in p-GSK3β (Figure 3D, P < 0.001 vs non-treated group). Furthermore, with a specific GSK-3β inhibitor BIO produced a concentration-dependent and significant decrease in tumour cell growth (Figure 3F, P < 0.001 vs non-treated group).

DISCUSSION

In the present study, we investigated the role of KOR in NSCLC cells using gefitinib-sensitive HCC827 and gefitinib-resistant H1975 cells. We found that KORs were highly expressed in both cell lines. Under these conditions, addition of the selective KOR agonist U50,488H to either HCC827 or H1975 cells produced a concentration-dependent decrease in tumour cell growth. Although some of the doses of U50,488H were relatively high, these effects were abolished by co-treatment with the selective KOR antagonist nor-BNI. These results support the idea that U50,488H can pharmacologically act on KORs to decrease tumour growth. Additionally, the inhibition of tumour growth by gefitinib in HCC827 cells was further enhanced by co-treatment with U50,488H. These findings suggest that the stimulation of KOR may provide unique opportunities for the prevention and treatment of NSCLC.

GSK3β is a multifunctional serine/threonine kinase that phosphorylates and thereby regulates the functions of many metabolic, signaling, and structural proteins and transcriptional factors (Grimes and Jope, 2001). EGF can inactivate GSK3β, leading to the degradation of c-Myc and β-catenin, which are overexpressed in tumour cells. Furthermore, the tumour suppressor p53 can be inactivated because of inactive GSK3β. It has been reported that the progressive inactivation of GSK3β, which is related to the increase in phosphorylation of GSK3β, is critical for the progression of lung cancer (Tian et al., 2006). In this study, treatment of H1975 cells with U50,488H produced a significant decrease in the phosphorylation of GSK3β. It has been recognised that activated protein kinase A (PKA) leads to phosphorylation of GSK3β, which is responsible for the decrease in the phosphorylation of GSK3β (Fang et al., 2000), whereas activated JNK increases GSK3β activity (Hu et al., 2009). It should be noted that the stimulation of KOR suppresses cAMP production through Gi proteins, which leads to the inactivation of PKA (Tso and Wong, 2003). Furthermore, the stimulation of KOR invokes the JNK cascade (Kam et al., 2004). Although the exact mechanism of
KOR-mediated GSK3β activation remains unclear at this time, we propose that the stimulation of KOR may activate GSK3β through inhibition of the cAMP/PKA pathway and/or activation of the JNK pathway in NSCLC, resulting in the prevention of cancer.

In conclusion, the present results suggest that stimulation of KOR reduces the growth of NSCLC cells through the activation of GSK3β. Furthermore, KOR agonist might be a valuable candidate for preventing gefitinib-resistant NSCLC.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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