Baclofen, a GABAB receptor agonist, inhibits human hepatocellular carcinoma cell growth in vitro and in vivo

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Abstract

Gamma aminobutyric acid (GABA) has been reported to affect cancer development, but the activation of its type B receptor (GABABR) has shown contradictory effects on the progress of human carcinoma. In this study, we investigated the antitumor effect of the GABABR agonist baclofen (Bac) on growth of human hepatocellular carcinoma (HCC) in vitro and in vivo. We found Bac induced G0/G1 phase arrest which was associated with down-regulation of intracellular cAMP level, and up-regulation of p21WAF1 protein expression as well as its phosphorylation level. These in vitro effects could be abrogated by pretreatment with the specific GABABR antagonist phaclofen (Pha). Moreover, systemic administration of Bac significantly suppressed Bel-7402 xenograft tumor growth. Our data support the inhibitory effect of GABABR activation on HCC development, which would raise the possibility to develop Bac as a therapeutic drug for the treatment of HCC.

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Introduction

Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS). It acts at either ionotropic GABA A or GABA C receptors (GABAAR or GABACR) or metabotropic GABAB receptors (GABABR). Outside the CNS, functional GABA receptors are expressed in a variety of normal non-neuronal tissues (e.g. lung, liver and gastrointestinal tract) (Billington et al., 2001), as well as some malignant cells (e.g. mammary gland, colon and hepatic tumor cells) (Ortega, 2003; Chapman et al., 1993; Opolski et al., 2000; Minuk, 2000). GABA A and GABA C receptors are intrinsic Cl⁻ channels, while GABAB receptors belong to the G protein-coupled receptor (GPCR) superfamily. So far, two GABAB receptor subunits, GABAB receptor 1 (GABABR1) and GABAB receptor 2 (GABABR2), have been identified. Unlike other members of the GPCR superfamily, functional GABAB receptors require the formation of a heterodimer with GABABR1 and GABABR2 subunits (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998).

GABA receptors have been implicated as playing roles in synaptic inhibition, convulsion, pain, depression and cognition (Watanabe et al., 2002). Recent reports extend these observations by providing evidence that the receptors play an inhibitory role in mammary cancer growth and as negative regulators of tumor cell migration (Opolski et al., 2000; Sun et al., 2003; Fava et al., 2005; Joseph et al., 2002; Drell et al., 2003; Entschladen et al., 2002). In parallel to these observations, GABABR agonist Bac treatment was demonstrated to reduce the incidence of some carcinogen-induced gastrointestinal cancers in rats (Tatsuta et al., 1990, 1992). However, in contrast to its suppressive effects on cancer development, some investigators have provided opposite evidence that GABABR activation promoted human prostate cancer cell migration (Azuma et al., 2003).
far, the role of GABABR activation in cancer development remains controversial.

Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal forms of cancer in Asia and Africa (Blum, 2005). Although in recent years the major etiologies and the risk factors for HCC development have been well defined and some of the multiple steps involved in hepatocarcinogenesis have been elucidated, the HCC survival rate has not improved during the last three decades (Blum, 2005). In this study, we aim to investigate the antitumor effect of Bac on human HCC carcinoma growth, which will help to elucidate the role of activation of GABABR on HCC development.

Materials and methods

Materials and cell lines

RIA kits for the determination of intracellular cAMP levels were from Amersham Biosciences. Bac and its antagonist phaclofen (Pha) were bought from Sigma-Aldrich, and dissolved in 0.9% NaCl. The antibodies for GABABR1, GABABR2, p21WAF1 and phospho-p21WAF1 were from Santa Cruz. The human hepatocarcinoma cell line Bel-7402 was from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), HepG2 and HuH-7 cell lines were from American Type Culture Collection (ATCC). All these cells were grown in DMEM (GIBCO-BRL) supplemented with 10% fetal bovine serum (JRH Biosciences), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma).

RNA extraction and RT-PCR

Total RNA was extracted using Trizol and isolated according to the procedure supplied by the manufacturer (Invitrogen). Reverse transcription was carried out according to the manufacturer’s instructions (GIBCO-BRL). The first strand of the cDNA was generated from 5 μg of total RNA using oligo-dT primer and superscript II reverse transcriptase (GIBCO-BRL). Nucleotide primers were prepared based on the sequences of GABABR1, GABABR2 and β-actin. The sequences of these oligonucleotide primers were as follows: Primer for GABABR1 (280 bp), 5′-TGA AAC GCA GGA CAC CAT GA-3′ (forward) and 5′-TCA CTT GTA AAG CAA ATG TAC T-3′ (reverse). Primer for GABABR2 (850 bp), 5′-ACC ATA AGG TTC CAG GGG TC-3′ (forward) and 5′-AGG CAG AGA GTG ATG GTG CT-3′ (reverse). Primer for β-actin (713 bp): 5′-TGC TAT CCC TGT ACG CCT CT-3′ (forward); antisense, 5′-CTA GAA GCA TTT GCG GTG GA-3′ (reverse). The PCR was performed initially by denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min (for GABABR2, duration of 90 s), and a final extension step at 72°C for 10 min. Amplified DNA fragments were electrophoretically fractionated on 1% agarose gels, cloned and sequenced.

Immunoblotting

Cells were collected and resuspended in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH2PO4, 10 mM sodium molybdate, 2 mM sodium orthovanadate, 20 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 6 mM diethiothreitol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride) and sonicated six times (30-second bursts). Samples were subjected to SDS polyacrylamide gel electrophoresis and transferred to Immobilon membranes (Bio-Rad). After blocking of nonspecific sites, filters were reacted with primary antibodies and corresponding horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence detection system (Pierce).

Cell proliferation assay

Bel-7402, HuH-7, and HepG2 cells were seeded into 96-well plates (10,000 per well) in a final volume of 200 μl medium. After a 24-hour attachment, cells were incubated with Bac (25, 50 and 100 μM) in the absence or presence of Pha (100 μM) for indicated time intervals. During this time, the culture medium was replaced with fresh medium every 24 h. After treatment, cell proliferation was evaluated by 3-(4, 5-dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)H-tetrazolium, inner salt (MTS) assay. The OD values were measured at 490 nm with a microplate spectrophotometer (Molecular Devices).

Cell cycle analysis

Cells were synchronized by incubation in serum-free medium for 12 h and then in serum-supplemented medium containing Bac (100 μM) in the absence or presence of Pha (100 μM) for 24 h. Cells were harvested, washed twice with ice-cold PBS and fixed in 75% ethanol at −20°C overnight. Then cells were washed with PBS, incubated with 100 μg/ml RNase A at 37°C for 30 min, stained with 25 μg/ml propidium iodide solution and analyzed with flow cytometry (FCM; BD FACScalibur). The cell cycle was analyzed by Modfit software (BD Biosciences).

Intracellular cAMP detection

After trypsinization, cells were incubated at 37°C for 1 h to regenerate membrane proteins damaged by trypsin. Cells were then incubated with 0.2% BSA (basal) or Bac (100 μM) with or without a 10-minute preincubation with Pha (100 μM) at room temperature for 10 min. After freezing and thawing, the cells were pelleted by centrifugation, and aliquots of the supernatant were assayed for cAMP by radioimmunoassay (PerkinElmer) according to the manufacturer’s protocol.

Subcutaneous xenograft of Bel-7402 cells in nude mice

Male Balb/c nude mice (18–22 g at 4–6 weeks of age) were from the Experimental Animal Center of Shanghai Cancer Institute (Shanghai, China). Cells (2 × 10⁶ cells in 200 μl of serum-free medium) were subcutaneously injected into the flank of the Balb/c nude mice. When tumors had reached a volume of
about 0.1 cm³, the mice were given an intraperitoneal injection of Bac 30 mg/kg or vehicle every day. Tumor growth was monitored every 5 days by measuring two perpendicular diameters with calipers. The tumor volumes were calculated using the equation \( V = \frac{D \times d^2}{2} \), where \( V \) (cm³) is the tumor volume, \( D \) is the longest diameter, and \( d \) is the shortest diameter. Mice were sacrificed when the tumor volumes reached at about 2.5 cm³ in the control group. This study was carried out in accordance with Chinese government guidelines.

Statistical analysis

Data are expressed as the means±S.D. A \( P \)-value less than 0.05 was considered statistically significant.

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![Figure 1](image1.png)

**Fig. 1.** The expression of GABABR1 and GABABR2 subunits in hepatocellular carcinoma cell lines. (A), The messenger RNA expression for GABABR1 and GABABR2 subunits in Bel-7402, HepG2 and HuH-7 cell lines by RT-PCR. (B), Western blot analysis for detecting the protein expression of GABABR1 and GABABR2 subunits in Bel-7402, HepG2 and HuH-7 cells.

![Figure 2](image2.png)

**Fig. 2.** Bac inhibited the growth of hepatocellular carcinoma cells. MTS assay was used to detect cell growth. (A), The effect of Bac on Bel-7402 cell growth. (B), The effect of Bac on HuH-7 cell growth. (C), The cells were incubated with Bac (100 μM) in the absence or presence of 100 μM Pha and observed at the indicated time intervals. Proliferation index was calculated as the ratio of the absorbance of cells incubated with Bac or Bac combined with Pha in comparison with the controls. All experiments were repeated in triplicate and the standard deviations are indicated. * or # represent \( P < 0.05 \) versus Bac + Pha group; **, \( P < 0.001 \) versus Bac + Pha group. (D), The effect of 100 μM Bac on cell cycle distribution of Bel-7402 cells with or without 100 μM Pha. Representative data from three independent experiments are presented.
Results

Detection of GABABR subunits in HCC cells

The HCC cell lines Bel-7402, HepG2 and HuH-7 were examined for presence of GABABR subunits at both mRNA and protein levels. As shown in Fig. 1A, both GABABR1 and GABABR2 mRNA were detectable in these cell lines. No significant differences of GABABR1 or GABABR2 mRNA expression level were found among the various cell lines. In parallel, all these cell lines were examined for GABABR1 and GABABR2 protein expression, and no significant differences were found (Fig. 1B).

Bac inhibits cell proliferation and induces cell cycle arrest at G0/G1 phase

The effect of Bac on cell proliferation was determined by MTS assay. As illustrated in Fig. 2A and B, Bac dose-dependently suppressed cell growth in both cell lines. Specifically, after a 4-day exposure, the cell viability in the 100 μM Bac-treated group fell by approximately 41.8% and 64.5% in Bel-7402 (Fig. 2A) and HuH-7 (Fig. 2B) cells, respectively. Bac at 100 μM was thus taken as the effective dose in the following experiments. Pha, an antagonist of GABABR, was used to determine whether Bac-induced cytotoxicity was mediated by GABABs. Treatment of HCC cells with Pha (100 μM) alone did not suppress cell proliferation (data not shown), whereas when given together with Bac (100 μM), Pha significantly reversed Bac-induced growth inhibition (Fig. 2C). To further assess the effect of Bac on cell proliferation, cell cycle distribution was measured in Bac-treated Bel-7402 cells. As shown in Fig. 2D, Bac (100 μM) treatment for 24 h induced G0/G1 phase arrest by 62.5%, which was mostly reversed by co-treatment with Pha (100 μM).

Bac-induced growth inhibition pattern correlates with down-regulation of intracellular cAMP level and up-regulation of p21WAF1

To determine how Bac affect HCC cell proliferation, we examined cAMP level and p21WAF1 expression as well as its phosphorylation status in Bac-treated cells. As illustrated in Fig. 3A, Bac (100 μM) exposure significantly decreased intracellular cAMP level by 39.3% and 55.9% in Bel-7402 and HuH-7 cells, respectively. Such effects of Bac on cAMP level were effectively reversed by Pha (100 μM) co-treatment in both cell lines (Fig. 3A). The level of p21WAF1 expression and its phosphorylation status were determined by western blotting. In Bac (100 μM) treated Bel-7402 cells, we detected up-regulation of p21WAF1 protein and its phosphorylation levels (Fig. 3B).

Baclofen suppressed Bel-7402 xenograft tumor growth in vivo

To further explore the potential role of GABABR in HCC development, we examined the effect of Bac on Bel-7402 xenograft growth in nude mice. Antitumor efficacy was measured as tumor growth inhibition rate, defined as \(1 - \left(\frac{T}{C}\right) \times 100\)%, where T and C represent the mean tumor volumes in the treated and untreated control groups, respectively. The individual group comparisons were evaluated by two-way ANOVA. We observed that the administration of Bac (30 mg/kg/d) significantly suppressed tumor growth (Fig. 4A). The inhibition rates in the Bac-treated group were 32.57%, 39.24% and 45.47% on 20, 25 and 30 days of administration, respectively \((P<0.05; \text{Fig. 4A})\). During the 30-day treatment, no abnormal deaths were observed. Compared to their initial weights, the maximal body weight loss reached about 12% in Bac-treated mice. No significant difference was found in the mean body weight between the two groups (Fig. 4B).

Discussion

Outside the CNS, the GABAergic system has been identified in a variety of normal and malignant tissues (Watanabe et al., 2002; Opolski et al., 2000; Sun et al., 2003; Chapman et al.,...
1993; He et al., 2001; Isomoto et al., 1998; Glassmeier et al., 1998). Most of the tumors studied to date are depolarized relative to adjacent non-tumor tissue, a finding in keeping with decreased GABAergic activity (Minuk, 2000; Nicholson et al., 2001; Mazurkiewicz et al., 1999). The strategies for enhancing GABAergic activity resulted in growth inhibition of various transformed cell lines (Minuk, 2000), with the process mediated by GABA receptors. In human HCC, GABAA-beta3 was down-regulated at both mRNA and protein level compared to adjacent non-tumor tissues (Minuk et al., 2007). Restoration of GABAA function by transfecting with GABAA-beta3 gene or using the GABAA agonist muscimol, demonstrated to increase the membrane potential differences and inhibit proliferation of malignant cells (Sun et al., 2003; Zhang et al., 2000). However, the role of GABABR in HCC development has remained less well known.

In the present study, we found GABABR1 and GABABR2 were commonly expressed in HCC cell lines (Fig. 1). Treatment of the cells with the GABAB agonist Bac significantly suppressed cell proliferation with G0/G1 phase arrest (Fig. 2), while it did not induce cell death even at doses much higher than 100 μM (data not shown). Bac at 100 μM was chosen as the effective dose in in vitro study. This dose was demonstrated to affect matrix metalloproteinase 3 production in human prostate cancer cell line C4-2 (Azuma et al., 2003). The inhibitory effect of Bac on cell growth could effectively be reversed by co-treatment with the GABAB antagonist Pha, suggesting that Bac-induced growth inhibition was exerted in a GABAB-dependent manner. In neuronal cells, GABAB receptor activation reduced cAMP level, impeded PKA activation and thus physiological functions (Bormann, 2000; Billinton et al., 2001). In this study, we found Bac-induced G0/G1 arrest pattern was in parallel with its effect on down-regulation of cAMP (Fig. 3A), implying that cAMP might also be an important signal in GABAB-receptor mediated tumor cell growth. In addition, Bac-induced G0/G1 arrest was demonstrated to be related to the effect of Bac on p21WAF1 expression level and to its phosphorylation status (Fig. 3B). p21WAF1 is a key regulator of the cell cycle and acts as a universal inhibitor of cyclin-dependent kinases (Xiong et al., 1993; Niculescu et al., 1998). p21WAF1 expression and its phosphorylation status negatively regulate G1/S transition (Niculescu et al., 1998). The up-regulated p21WAF1 expression and phosphorylation levels induced by Bac-treatment could be partially suppressed by Pha co-treatment (Fig. 3B), suggesting this process might also be GABAB-dependent. The HCC growth suppression effect of Bac was strongly supported by the data obtained from in vivo study on the model of Bel-7402 xenograft nude mice (Fig. 4A). In this model, Bac was administered at a single dosage of 30 mg/kg every day for 30 days. The results suggested Bac at this dose is effective in suppressing tumor growth (Fig. 4A) and without causing ‘toxic’ effects (Fig. 4B). Usually treatment producing >20% lethality and/or 20% net body weight loss are considered ‘toxic’ (Liu et al., 2006). A previous study suggested that a longer Bac application (e.g. 8 mg/kg for 52 weeks) might suppress appetite and thus cause significant body weight loss in some animals (Tatsuta et al., 1996). In this study, no animals died and the maximal body weight loss was less than 20% in the Bac-treated group; we therefore considered Bac as an effective and relatively safe potential drug for treating HCC.

Conclusion

In summary, this study shows that Bac suppresses tumor cell growth both in vitro and in vivo. The inhibition of cAMP, the down-regulation of p21WAF1 protein level and the reduction of p21WAF1 phosphorylation may contribute to the anti-tumor effect of Bac. Moreover, the anti-tumor effect of Bac could be reversed by Pha, suggesting that Bac inhibits tumor cell growth through activating GABAB receptors. Our observations indicate that Bac, and perhaps other GABAB agonists, represent a novel targeted approach for treating HCC.

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References


