In vitro and *in silico* investigation of the critical role of $GABA_B$ receptor activation in human breast cancer and its natural agonists

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Abstract. γ-aminobutyric acid type B ($GABA_B$) receptors, a class of G protein-coupled receptors, serve pivotal roles in modulating cellular signaling pathways across both neural and peripheral tissues, although their underlying mechanisms involved in cancer progression have yet to be fully elucidated. The aim of the present study was to investigate the effect of $GABA_B$ receptor activation on the migration, invasion, clonogenicity, proliferation and epithelial-mesenchymal transition (EMT) of the MDA-MB-231 human breast cancer cell line. To meet this aim, MDA-MB-231 cells were treated with various concentrations of the $GABA_B$ receptor agonist, baclofen. Treatment with baclofen caused a significant inhibition of cell migration, invasion and clonogenicity in a dose-dependent manner. Moreover, baclofen treatment led to a downregulation of vimentin expression, whereas the expression of β-catenin was upregulated, indicating suppression of EMT in MDA-MB-231 cells. Notably, the phosphorylation levels of Akt and ERK1/2 remained unaltered, suggesting that the inhibitory effects mediated by baclofen on EMT were not mediated via either the phosphoinositide 3-kinase/Akt or the MAPK pathways. To identify potential natural $GABA_B$ receptor agonists, molecular docking techniques were employed using the MPD3 database. This *in silico* approach revealed three compounds (PubChem identification, 450432, 6448 and 6057) as promising candidates, which all exhibited critical hydrogenbond interactions with the $GABA_B$ receptorbinding site. These compounds may serve as lead compounds for developing novel, naturally derived $GABA_B$ receptor agonists. Taken together, the findings demonstrated that $GABA_B$ receptor activation via baclofen inhibits multiple aggressive phenotypes in MDA-MB-231 breast cancer cells. Moreover, these results offer promising avenues for developing $GABA_B$ receptor agonists as a therapeutic strategy in breast cancer treatment.

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Abbreviations: EMT, epithelial-mesenchymal transition; ZEB1, zinc finger E-box binding homeobox 1; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; GABA, γ-aminobutyric acid; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Key words: $GABA_B$ receptor, EMT, metastasis, breast cancer, molecular docking, signal transduction

Introduction

Breast cancer is a widespread metastatic disease among women, which is exceeded only by lung cancer, comprising ~29% of all cases of female cancer in the USA in 2001 (1). A previous study revealed that in the USA 1/8 women will develop invasive breast cancer during their lifetime, compared with ~1/800 men. As of 2023 there were 4 million cases of breast cancer among women in the USA, including all women who were either still receiving treatment or had already finished the treatment (2). A previous study, including the study by Eccles *et al* (3), have focused on the epidemiology and basic biology of breast cancer therapy, which have

undergone several revisions due to the detection of predictive biomarkers that have enabled the use of a wider range of application therapies for this disease. Memon *et al* (4) identified several primary risk factors associated with breast cancer development, including family history, hormone replacement therapy during menopause, and nulliparity or delayed childbearing. Inherited genetic mutations, including those in breast cancer susceptibility genes 1 and 2, as well as other genes such as partner and localizer of BRCA2a, ataxia telangiectasia mutated, checkpoint kinase 2 and tumor protein p53, have also been shown to contribute to 5-10% of breast cancer cases (5).

Wang *et al* (6) demonstrated that numerous signaling pathways mediate epithelial-mesenchymal transition (EMT) and cancer progression. KudoSaito *et al* (7) reported that Snail family transcriptional repressor 1 induces EMT, leading to a promotion of invasion and acceleration of cancer metastasis. Zinc finger E-box-binding homeobox 1 (ZEB1) also acts as an inducer of EMT in several types of human tumors, including breast, colorectal, pancreatic and non-small cell lung cancers, where ZEB1 increases the rates of invasion or metastasis of the cancer (8). In addition, a previous study by Tania *et al* (9), have shown that EMT may be induced by ZEB proteins using mouse models and primary human carcinomas, which thereby increased tumor aggressiveness and metastasis. Furthermore, the phosphoinositide 3-kinase (PI3K)/Akt pathway is considered a critical signaling pathway, which serves important roles in numerous cellular activities, including development and proliferation, and is also frequently associated with different types of human cancer (10), including breast cancer. The PI3K/Akt pathway may be activated by Gprotein-coupled receptors through receptor tyrosine kinases located on the cell surface, such as the epidermal growth factor receptor, which is often found dysregulated on the surface of breast cancer cells (11).

γ-aminobutyric acid (GABA) is an important neurotransmitter that acts as an inhibitor in the central nervous system. GABA acts on two different types of receptors, namely ionotropic and metabotropic receptors, based on their pharmacological and physiological properties, thereby eliciting powerful inhibitory effects. Ionotropic GABA receptors (namely, $GABA_A$ and $GABA_C$ receptors) are ligand-gated chloride channels that mediate rapid inhibition responses, whereas metabotropic GABA receptors ($GABA_B$ receptors) are G-protein-coupled receptors that mediate slow inhibitory signals (12). $GABA_B$ receptors consist of two isomers, $GABA_B$ receptor 1 and $GABA_B$ receptor 2. Previous studies have reported that the $GABA_B$ receptors are involved in tumor progression (13,14). A study by Tatsuta *et al* (15) was the first to identify the association between $GABA_B$ receptor activation and cancer. It was demonstrated that prolonged administration of GABA at high doses and the $GABA_B$ receptor agonist baclofen significantly decreased the incidence and number of gastric cancers in Wistar rats. This inhibitory effect on gastric carcinogenesis occurred via $GABA_B$ receptors, not $GABA_A$ receptors, as demonstrated by the lack of effect with muscimol (a $GABA_A$ agonist). The aforementioned study also showed that both GABA and baclofen decreased cell proliferation in antral mucosa and increased serum gastrin levels, suggesting these mechanisms may contribute to their anti-cancer effects. $GABA_B$ receptor 1 is widely expressed in a range of cancer tissues and cancer cells, and it is closely associated with the occurrence and development of pancreatic, liver, colon and ovarian cancer (16). Kanbara *et al* (17) demonstrated that the $GABA_B$ receptor antagonist CGP 35348 has antitumor effects in the high-grade chondrosarcoma cell line OUMS27 and regulates proliferation through apoptotic pathways in high-grade chondrosarcoma cells. Another study reported that downregulation of the mRNA expression level of $GABA_B$ receptor can inhibit the proliferation of ovarian cancer cells (16). Furthermore, $GABA_B$ receptor 1 has been shown to promote the secretion of the peptide gastrin in neuroendocrine cells involved in prostate cancer progression. This effect leads to enhanced invasive potential of prostate cancer cells through GRP receptor activation, increased cell migration and creation of a more aggressive cancer phenotype, particularly in tumors with low levels of androgen receptor expression (18). Previous studies have also reported that $GABA_B$ receptors regulate the proliferation of colorectal cancer cells via the GSK-3β/NF-κB signaling pathway (18). In addition, Zhang *et al* (19) studied the role of GABA in breast cancer metastasis and reported that GABAergic signaling facilitates breast cancer metastasis by promoting ERK1/2-dependent phosphorylation and increasing matrix metallopeptidase 2 (MMP-2) expression levels in 4T1 mouse mammary carcinoma and MCF-7 human breast cancer cells. However, the effects of $GABA_B$ receptor activation may be cell line-dependent, as our preliminary observations with MDA-MB-231 cells suggested potentially different outcomes compared to those reported in 4T1 and MCF-7 cell lines.

The hypothesis of the present study is that $GABA_B$ receptor activation may inhibit metastatic behavior in breast cancer cells through modulation of EMT-associated pathways.

To test this hypothesis, the present study evaluated the effects of baclofen-mediated $GABA_B$ receptor activation on multiple phenotypes of MDA-MB-231 breast cancer cells. Furthermore, computational approaches were used to identify natural compounds with structural similarity to baclofen that could potentially function as $GABA_B$ receptor agonists. Through these experimental and computational approaches, the present study investigated the potential role of $GABA_B$ receptor modulation in breast cancer therapy.

Materials and methods

Cell culture and cell lines. Triple-negative MDA-MB-231 cells (Thermo Fisher Scientific, Inc.) were cultured at 37˚C for 48 h and subsequently washed carefully with PBS to eliminate the dead cells and the culture debris. Cells were then treated with 20% trypsin at 37˚C for several min. L-15 growth medium (5 ml; HyClone; Cytiva) was subsequently added and thoroughly mixed using a pipette to separate the cells. The cell suspension then underwent centrifugation for 1,200 x g for 5 min at 4˚C. The supernatant was discarded and resuspended in fresh L-15 medium (1 ml). Aliquots (200 μl) of the cells were then placed in the culture plate with 10 ml L-15 medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin. Cultures were incubated at 37˚C under standard conditions.

Treatment of the cells with $GABA_B$ receptor agonist. The $GABA_B$ receptor agonist baclofen (Tocris Bioscience) was

dissolved in water to form a stock solution (concentration, 10 mM) and stored at -20˚C until use. Cells ($2x10^4$) were seeded in 35-mm culture plates with L-15 medium containing 10% FBS. After 24 h, the medium was replaced with L-15 medium containing 1% FBS to induce starvation. Cells were subsequently treated with various concentrations of baclofen (0, 25, 50, 100 and 200 μM) for 24 h at 37˚C. These concentration ranges were selected based on previous studies investigating $GABA_B$ receptor activation in breast cancer cells (16,19).

Transwell migration and invasion assay. MDA-MB-231 cells were cultured in the aforementioned conditions in L-15 medium supplemented with 10% FBS. Migration and invasion assays were performed using 24-well Transwell chambers with 8 μm pore-size membranes. For the migration assay, 700 μl serum-free L-15 medium was added to the lower chamber. The upper chamber received 200 μl serum-free medium, followed by an incubation at 37˚C for 30 min to equilibrate the membrane and establish optimal conditions for cell attachment prior to adding the cell suspension. Subsequently, $2x10^4$ MDA-MB-231 cells in serum-free medium containing different concentrations of baclofen (0, 25, 50, 100 or 200 μM) were added to the upper chamber. The lower chamber medium was then replaced with L-15 medium containing 10% FBS and 100 U/ml penicillin-streptomycin. Cells were subsequently incubated at 37˚C for 24 h in an atmosphere lacking CO_2. Following this incubation, non-migrated cells were removed from the upper surface of the membrane, whereas migrated cells were fixed with 4% formaldehyde for 5 min at room temperature, permeabilized with 100% methanol for 20 min and stained with 1% crystal violet (prepared in a solution of 20% methanol and 79% PBS) for 20 min at room temperature. The membranes were subsequently air-dried and observed under an inverted light microscope at x20 magnification. Cell counts were obtained from at least eight random fields. The invasion assay protocol was similar to that of the migration assay, with the exception that a Matrigel® layer (70-80 μl of a 1 mg/ml concentration; Corning Life Sciences) was added to the upper chamber, which was subsequently allowed to form a gel for 1 h at 37˚C prior to cell seeding. A total of $5x10^4$ cells were seeded in the upper chamber and the incubation period for the invasion assay was extended to 48 h.

MTT assay. MDA-MB-231 cells at 75% confluence were starved in serum-free L-15 medium for 24 h, followed by subsequent trypsinization of 20% for 3 min. Cells were gently centrifuged at 125 x g for 6 min at room temperature, and the pellet was subsequently resuspended in fresh L-15 medium. Cells (100 μl) were then seeded into 24-well plates and treated with baclofen at various concentrations (25, 50, 100 or 200 μM) for 3 days at 37˚C. DMSO (0.01%) served as a control. On day 3, the medium was removed, cells were washed twice with PBS and 500 μl 0.5 mg/ml MTT solution in Opti-MEM™ Reduced-Serum Medium (Thermo Fisher Scientific, Inc.) was added to each well. After 30 min incubation at 37˚C, the solution was treated with 500 μl DMSO and shaken for 10 min at room temperature. Finally, the optical density (OD) was measured at 570 nm, and cell viability was calculated using the following formula: Cell viability (%)=[(OD (sample)-OD (blank)/(OD (control)-OD (blank)] x100.

Western blot analysis. MDA-MB-231 cells were treated with various concentrations of baclofen (0, 25, 50, 100 and 200 μM) for 24 h at 37˚C. Cells were lysed in 100-150 μl RIPA buffer (Beyotime Institute of Biotechnology) supplemented with 10% protease inhibitor cocktail and Na_3VO_4, and lysates were centrifuged at 13,600 x g at 4˚C for 10 min. Protein concentrations were determined using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.) and subsequently diluted with 4X Laemmli buffer containing dithiothreitol and heat-denatured and 30 μg of protein per lane was loaded onto SDS-PAGE gels. Proteins were separated by SDS-PAGE 10% and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature, and incubated with the following primary antibodies: Anti-β-catenin (rabbit monoclonal; cat. no. #8480; 1:1,000; Cell Signaling Technology, Inc.), anti-vimentin (rabbit monoclonal; cat. no. #5741; 1:1,000; Cell Signaling Technology, Inc.), anti-phospho-Akt (rabbit monoclonal; cat. no. #4060; 1:2,000; Cell Signaling Technology, Inc.), anti-Akt (rabbit monoclonal; cat. no. #4691; 1:1,000; Cell Signaling Technology, Inc.), anti-phospho-ERK1/2 (rabbit monoclonal; cat. no. #4370; 1:2,000; Cell Signaling Technology, Inc.), anti-ERK1/2 (rabbit monoclonal; cat. no. #4695; 1:1,000; Cell Signaling Technology. Inc.) and anti-GAPDH (mouse monoclonal; cat. no. sc-32233; 1:2,000; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. After washing three times with TBST (10 min each), membranes were incubated with anti-rabbit IgG (cat. no. #7074; 1:5,000; Cell Signaling Technology, Inc.) and anti-mouse IgG (cat. no. #7076; 1:5,000; Cell Signaling Technology, Inc.) HRP-conjugated secondary antibodies for 1 h at room temperature. Following three additional TBST washes (10 min each), protein band intensities were visualized using ECL detection reagent (Super Signal West Pico PLUS; Thermo Fisher Scientific, Inc.) and were quantified using Image Lab 5.2 software (Bio-Rad Laboratories, Inc.)

Colony formation assay. A single-cell suspension of MDA-MB-231 cells was prepared and diluted to a concentration of 200 cells/ml. Aliquots (1 ml) of this suspension were seeded into each well of a 24-well plate and incubated at 37˚C for 24 h. The L-15 medium was subsequently replaced with fresh medium containing 10% FBS and baclofen at various concentrations (0, 25, 50, 100 and 200 μM). Cells were then incubated at 37˚C for 10 days in an atmosphere lacking CO_2. Subsequently, cells were fixed with 300 μl methanol for 15 min at room temperature, washed with PBS and stained with 100 μl 1% crystal violet (in a solution of 20% methanol) for 20 min at room temperature. Plates were gently washed with water and observed under an inverted light microscope at x10 magnification. Colonies were defined as a cluster of ≥50 cells. Colony-forming efficiency was quantified by counting the number of colonies in each well using ImageJ software (version 1.53a; National Institutes of Health) and was expressed as a percentage relative to the control group.

Molecular docking studies. Molecular docking studies were performed using the open-source program Auto Dock Vina version 1.1.2 (20). A crystal structure of the $GABA_B$ receptor complexed with agonists (PDB ID 4MS4) was downloaded from the PDB database (https://www.rcsb.org/) (21). All the

small, crystallized molecules were removed, and the structure was optimized by adding polar hydrogens and computing the Gasteiger charges. A dataset of 1,635 natural lead-like compounds was subsequently retrieved from the MPD3 database (https://www.bioinformation.info/) (22). A Tanimoto coefficient (TC) similarity search was then performed against this dataset using baclofen as a reference, with a cut-off of 0.5. The free chemical informatics software Open Babel version 2.4.1 (23) was used for this similarity search. Filtered compounds were optimized, again by adding polar hydrogens and computing Gasteiger charges. A grid box of size 25x25x25 Å (25 $Å^3$) was centered on the binding pocket. Finally, the docking results were analyzed based on the interactions between the docked molecules and the $GABA_B$ receptor.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software version 8.0 (GraphPad; Dotmatics). Data are presented as mean ± standard error of the mean from at least three independent experiments. One-way ANOVA analysis with Dunnett's post hoc test was used to compare multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

$GABA_B$ receptor activation inhibits cell migration. To investigate the effects of $GABA_B$ receptor activation on cell migration, migration assays were performed using MDA-MB-231 breast cancer cells treated with various concentrations of the $GABA_B$ receptor agonist, baclofen (Fig. 1). The results demonstrated that baclofen treatment significantly reduced cell migration compared with the control group. This inhibition was dose-dependent, with significant reductions observed at 25 ($P<0.001$), 50 ($P<0.0001$), 100 ($P<0.0001$) and 200 ($P<0.0001$) compared with the untreated control. The most pronounced inhibition was observed at 200 μM. This observed inhibition of cell migration in MDA-MB-231 cells following baclofen treatment suggests a crucial role for $GABA_B$ signaling in modulating metastatic potential.

$GABA_B$ receptor activation inhibits cell invasion. To investigate the effect of $GABA_B$ receptor activation on cell invasion, Transwell invasion assays using MDA-MB-231 cells treated with baclofen were performed (Fig. 2). The results revealed dose-dependent inhibition of cell invasion in response to baclofen treatment. A significant reduction in the number of invaded cells was observed at the lowest concentration of baclofen tested (25 μM; $P<0.001$). This inhibitory effect became more pronounced with increasing baclofen concentrations, showing enhanced significance at 50, 100 and 200 μM (all $P<0.0001$), indicating that a robust, dose-dependent association existed between $GABA_B$ receptor activation and a decreased invasive capability of the MDA-MB-231 cells. The observed inhibition of cell invasion in MDA-MB-231 cells following baclofen treatment also suggests a crucial role for $GABA_B$ signaling in regulating breast cancer cell metastasis.

$GABA_B$ receptor activation inhibits cell clonogenicity. To investigate the long-term effects of $GABA_B$ receptor activation on breast cancer cell survival and proliferation, colony

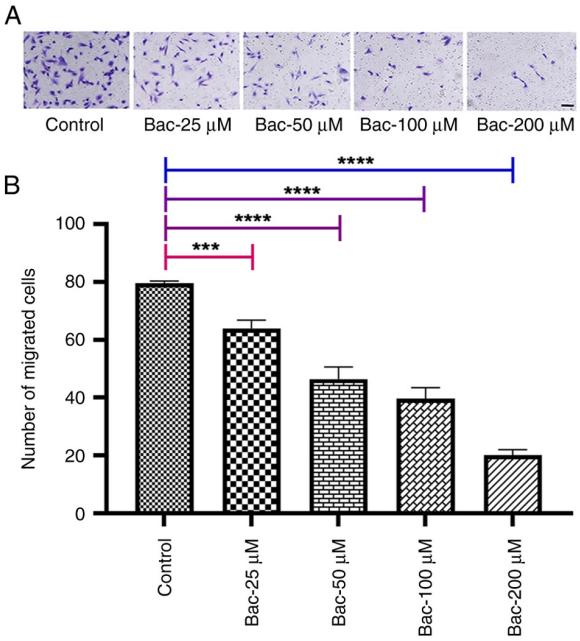


Figure 1. γ-aminobutyric acid B receptor activation affects the numbers of migrated cells. (A) MDA-MB-231 cells ($2x10^4$) were seeded into the upper chambers of the Transwell plates. Following 24 h baclofen treatment at different concentrations (0, 25, 50, 100 and 200 μM), the numbers of cells that had migrated were counted, and images were captured under the microscope at x20 magnification. (B) Data are shown as the mean ± standard error of the mean from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test. ***P<0.001 and ****P<0001 compared with the untreated control. Bac, baclofen.

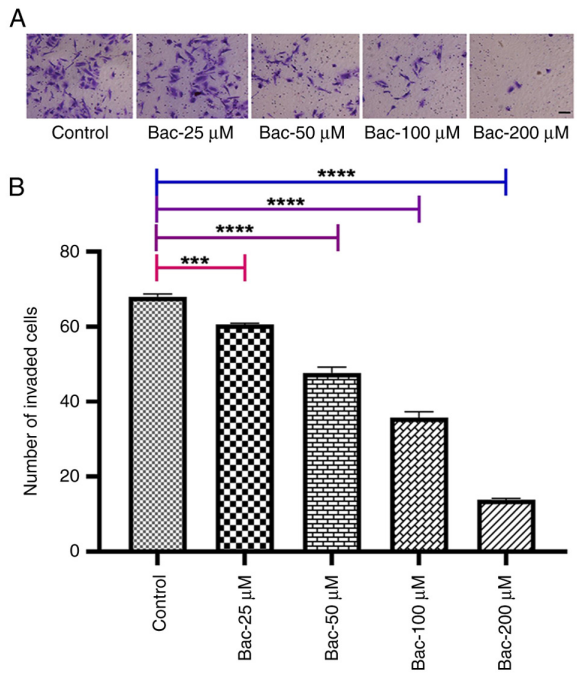


Figure 2. γ-aminobutyric acid B receptor activation affects the numbers of invaded cells. (A) Representative images of invaded MDA-MB-231 cells following baclofen treatment are shown. The upper chambers of the Transwell plates were pre-coated with 70 μl Matrigel for 1 h. MDA-MB-231 cells ($5x10^4$) were seeded in each upper chamber and treated with baclofen at various concentrations (0, 25, 50, 100 or 200 μM) for 48 h. Images were captured at x20 magnification. (B) Data are presented as the mean ± standard error of the mean from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test. ***P<0.001 and ****P<0001 compared with the untreated control. Bac, baclofen.

formation assays were performed. MDA-MB-231 cells were treated with various concentrations of baclofen (25-200 μM) and the ability of the cells to form colonies was evaluated over 10 days. The results demonstrated that $GABA_B$ receptor activation by baclofen significantly inhibited the clonogenicity potential of MDA-MB-231 cells in a dose-dependent manner (Fig. 3). Moreover, the lowest concentration of baclofen tested (25 μM) did not show a significant effect, whereas concentrations of 50 μM (P<0.01), 100 μM (P<0.001) and 200 μM (P<0.001) significantly progressively reduced colony formation capabilities. This inhibitory effect on colony formation, combined with the observation that baclofen did not significantly affect short-term cell viability in the MTT assay (Fig. 4), suggested that $GABA_B$ receptor activation preferentially impaired the cells' capacity for sustained proliferation rather than inducing immediate cytotoxicity.

Effect of $GABA_B$ receptor activation on MDA-MB-231 cell viability. The viability of the cells incubated with the specified concentrations of baclofen is shown in Fig. 4. In the present study, an MTT assay was performed to examine whether $GABA_B$ receptor activation could inhibit the viability of MDA-MB-231 cells. The MTT assay results demonstrated that $GABA_B$ receptor activation by baclofen did not significantly affect the viability of MDA-MB-231 cells across all tested concentrations. Even at the highest concentration of baclofen tested (200 μM), baclofen treatment exhibited no significant inhibitory effects on cell survival.

$GABA_B$ receptor activation modulates EMT markers in breast cancer cells. Subsequently, the effects of $GABA_B$ receptor activation on EMT markers in MDA-MB-231 breast cancer cells were investigated by examining the protein expression levels of β-catenin (a mesenchymal marker) and vimentin (an epithelial marker), with GAPDH serving as a loading control. Western blot analysis revealed concentration-dependent modulation of EMT markers following baclofen treatment. The protein expression levels of the EMT-associated genes βcatenin and vimentin were evaluated as shown in Fig. 5. While the lowest concentration of baclofen tested (25 μM) exhibited minimal effects (P>0.05), administering higher concentrations led to significant changes in EMT marker expression. β-catenin levels were significantly increased at 100 μM (P<0.01) and 200 μM (P<0.001), while vimentin expression was markedly decreased at these same concentrations (P<0.01 and P<0.01, respectively). Taken together, these results provide a molecular basis for understanding how $GABA_B$ receptor activation suppresses metastatic behavior in breast cancer cells.

Effects of activated $GABA_B$ receptors on the PI3K/Akt and Ras/Raf/MAPK signaling pathways. Subsequently, the potential involvement of the PI3K/Akt and Ras/Raf/MAPK signaling pathways in mediating the effects of the $GABA_B$ receptor in breast cancer cells were investigated. Using western blot analysis with phosphorylation-specific antibodies, the activation status of both Akt and ERK1/2 following baclofen treatment was examined. The results demonstrated that $GABA_B$ receptor activation by baclofen did not significantly alter the phosphorylation levels of either Akt or ERK1/2 in MDA-MB-231 cells (Fig. 6). These findings suggested that

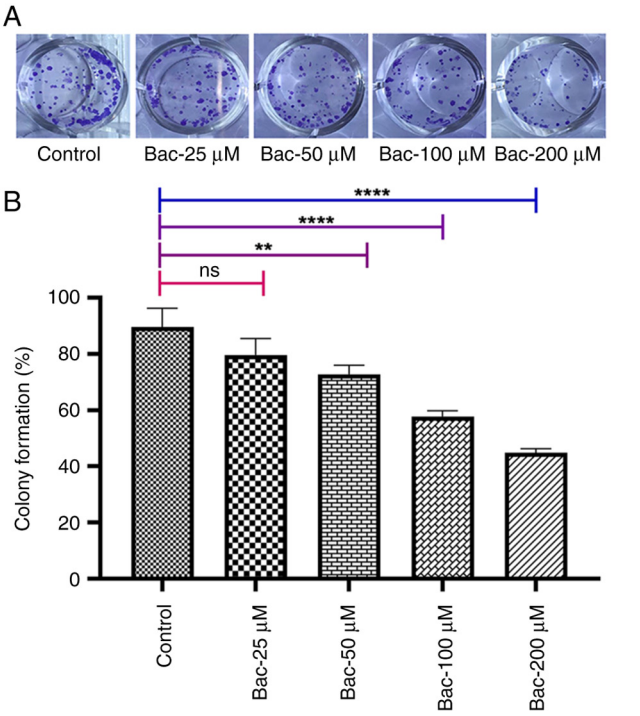


Figure 3. γ-aminobutyric acid B receptor activation inhibits cell survival and proliferation. (A) MDA-MB-231 cells (200 per well) were seeded in 24-well plates and treated with various concentrations of baclofen (0, 25, 50, 100 and 200 μM) for 10 days. Images were captured using a digital camera. (B) Data are shown as the mean ± standard error of the mean from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test. **P<0.01 and ****P<0001 compared with the untreated control. Bac, baclofen; ns, not significant.

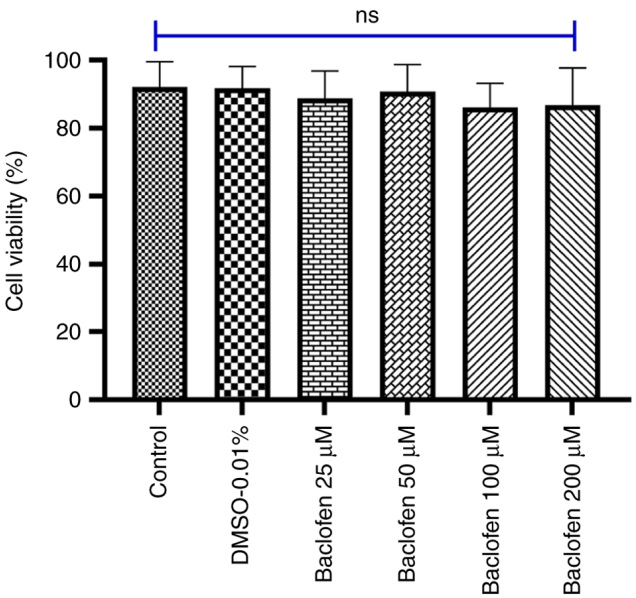


Figure 4. γ-aminobutyric acid B receptor activation exerts no effect on cell viability. MDA-MB-231 cells ($2x10^4$) were seeded into each upper chamber of a 24-well plate, and the cells were treated with baclofen at different concentrations (0, 25, 50, 100 and 200 μM) for 3 days. DMSO at a concentration of 0.01% was used as a control and the numbers of cells that survived were counted. The viability of the cells was found not to be affected following treatment with baclofen. The MTT assays were repeated three times, and each experiment yielded similar results. Data are shown as the mean ± standard error of the mean from three independent experiments. One-way ANOVA with Dunnett's post hoc test was performed to calculate statistical analysis of the mean compared with the untreated control. ns, not significant.

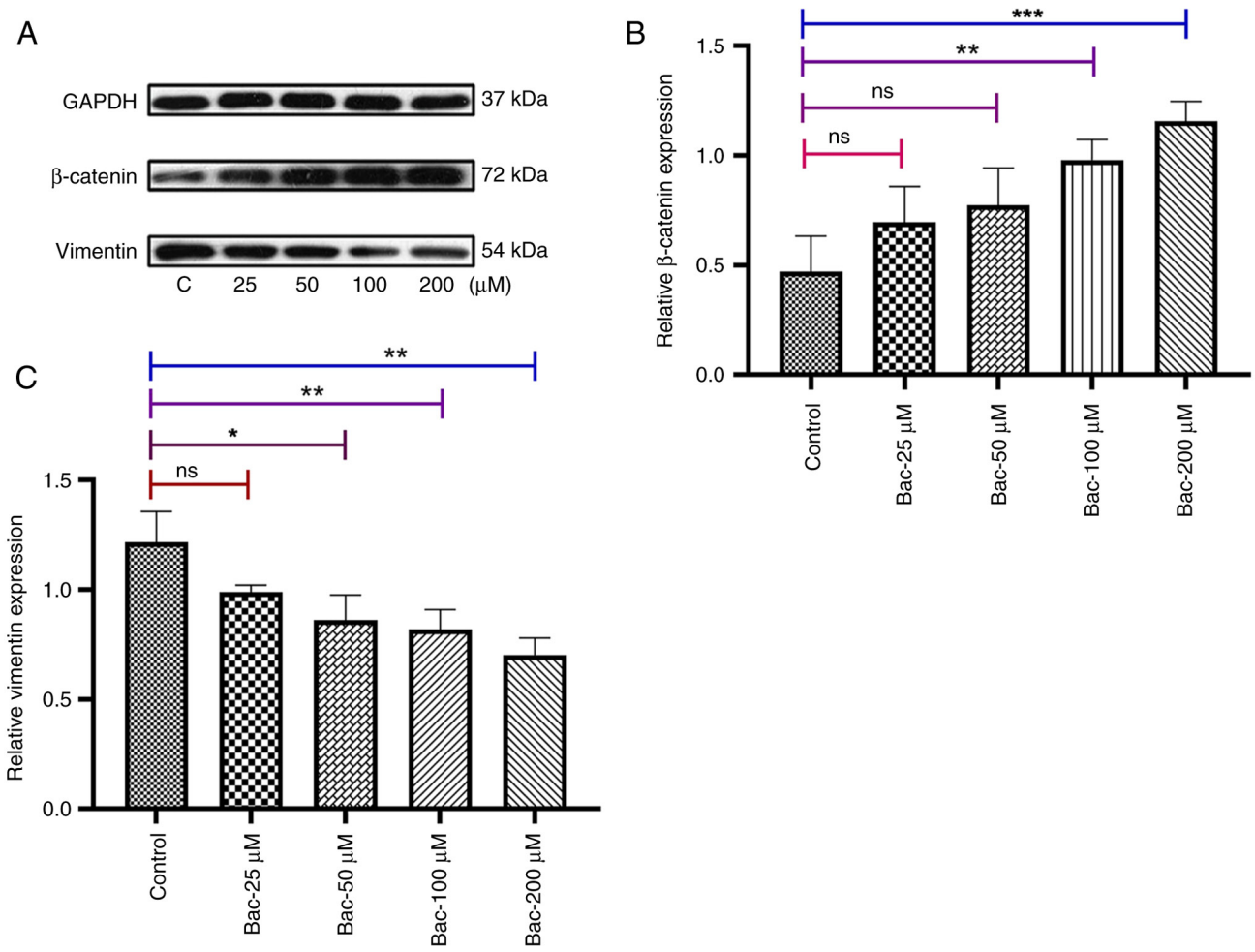


Figure 5. γ-aminobutyric acid B receptor activation modulates epithelial-mesenchymal transition markers in MDA-MB-231 breast cancer cells. (A) Representative immunoblots showing the expression of β-catenin and vimentin in MDA-MB-231 cells treated with various concentrations of baclofen. Following 24 h of serum starvation, cells were exposed to baclofen (0, 25, 50, 100, 200 μM) for 24 h. GAPDH served as a loading control. (B) Quantification of the expression of β-catenin relative to GAPDH is shown. Baclofen treatment induced dose-dependent increases in β-catenin levels, with significant upregulation observed at concentrations of 50, 100 and 200 μM. (C) Quantification of the expression of vimentin relative to GAPDH is shown. A significant decrease in vimentin expression was observed at a baclofen concentration of 100 and 200 μM. Data represent the mean ± standard error of the mean. from at least three independent experiments. Protein band intensities were quantified using ImageJ software. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test for comparisons with the control group. *P<0.05, **P<0.01 and ***P<0.001. ns, not significant; C, control, Bac, baclofen.

the anti-metastatic effects of $GABA_B$ receptor activation in MDA-MB-231 cells occured through mechanisms independent of the PI3K/Akt and Ras/Raf/MAPK signaling pathways, pointing to the involvement of alternative signaling cascades in mediating these effects.

Molecular docking and interaction analysis with $GABA_B$ receptor. Analysis of $GABA_B$ receptor binding identified five key residues (Ser-130, Ser-153, Tyr-250, Gly-151 and Glu-249) that form hydrogen bond interactions with baclofen, as shown in Fig. S1. Using a TC-based similarity search against 1,635 natural lead-like compounds from the MPD3 database, 17 compounds were identified with coefficients >0.4. Molecular docking analysis using Autodock Vina revealed that six compounds (PubChem identification, 450432, 31226, 441457, 6448, 10394 and 6057) formed three common hydrogen bond interactions with critical residues (including Ser-130, Ser-153 and Tyr-250) of the $GABA_B$ receptor (Table SI).

Further detailed binding interaction analysis, comparing both structural similarity scores (TC) and binding energies, identified three compounds with particularly favorable profiles (Table I). These compounds showed high structural similarity to baclofen (TC, 0.54-0.65) and comparable binding energies (-7.1 to -7.6 kcal/mol vs. -7.9 kcal/mol for baclofen). Specifically, compound 450432 (TC=0.54; binding energy=-7.1 kcal/mol) formed hydrogen bonds with Tyr-250, Gly-151, Ser-153 and Ser-130; compound 6448 (TC=0.52; binding energy=-7.6 kcal/mol) interacted with Ser-130, Ser-153, Tyr-250 and Gly-151; and compound 6057 (TC=0.65; binding energy=-7.2 kcal/mol) formed hydrogen bonds with Ser-130, Ser-153, Tyr-250 and Phe-249. The docked conformations and hydrogen bond interactions are illustrated in Figs. 7 and S2.

Discussion

The present study aimed to evaluate the role of $GABA_B$ receptor activation in breast cancer progression through complementary experimental and computational approaches. The results have provided insights into how $GABA_B$ receptor activation regulates breast cancer metastasis by selectively modulating

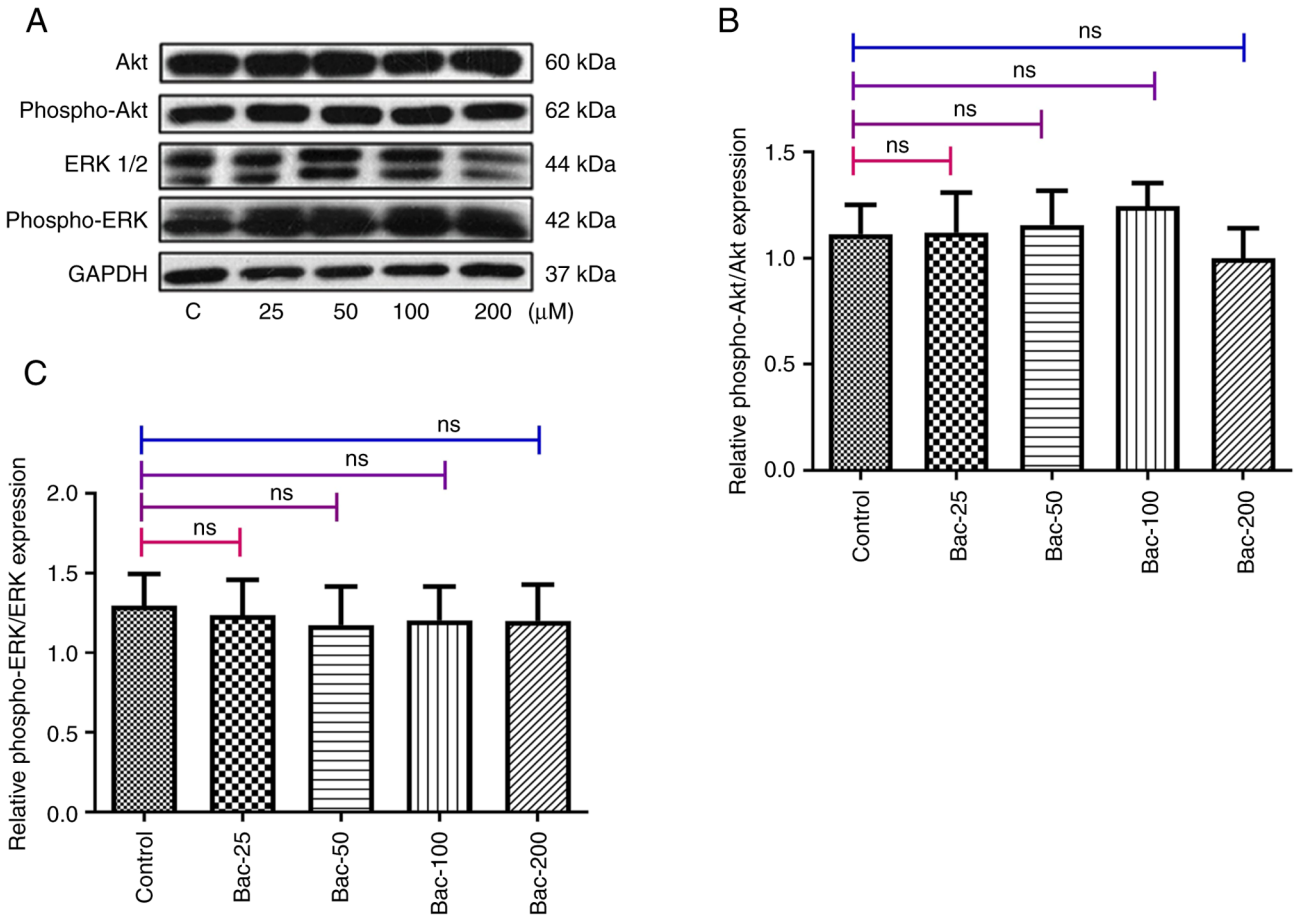


Figure 6. Effects of γ-aminobutyric acid B receptor activation on the P13K/Akt and Ras/Raf/MAPK signaling pathways. (A) After 24 h of treatment with different concentrations of baclofen (25, 50, 100 and 200 μM), the phosphorylation levels of Akt and ERK1/2 were found not to have been significantly altered. (B) Quantification of phospho-Akt/Akt. (C) Quantification of phospho-ERK/ERK. The experiments were repeated three times with similar results. Protein quantification was performed using ImageJ software. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test for comparisons with the control group. The data are shown as the mean ± standard error of the mean. ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; ns, not significant; Bac, baclofen; phospho, phosphorylated.

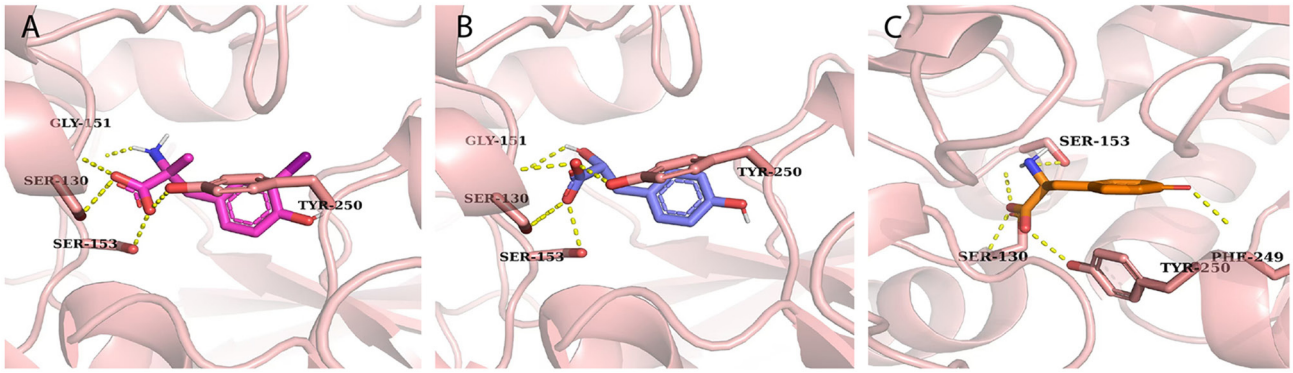
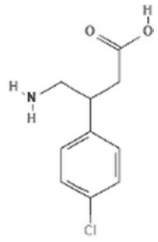
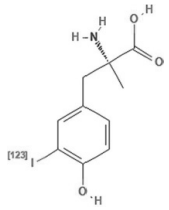
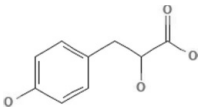
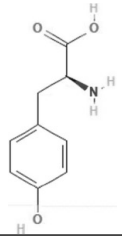


Figure 7. Illustration of the hydrogen bond interactions of the compounds with the γ-aminobutyric acid B receptor. PubChem identification of the compounds are as follows: (A) 450432, (B) 6448 and (C) 6057.

cellular behavior and EMT programming. The results demonstrate that $GABA_B$ receptor activation significantly inhibits metastatic phenotypes in MDA-MB-231 breast cancer cells, as evidenced by dose-dependent reductions in both cell migration and invasion following baclofen treatment. Furthermore, the rapid onset of these inhibitory effects, observed within 24 h baclofen administration, indicates direct modulation of cellular machinery controlling movement, including cytoskeletal reorganization pathways, cell-matrix adhesion molecules and actin polymerization regulators that are essential for cellular motility and invasiveness. These findings extend observations previously made by Gao *et al* (16) in ovarian cancer cells, who demonstrated that baclofen inhibited migration, invasion and EMT in SKOV3 cells. Lodewyks *et al* (24) reported that in

Table I. Chemical structures, TC similarity scores, binding scores and hydrogen bond interactions of baclofen and the three docked compounds with the γ-aminobutyric acid B receptor active site.

PubChem identification	Chemical structure	TC similarity score	Binding energy (Kcal/mol)	Hydrogen bond interactions
Baclofen		Reference	-7.9	Ser153, Ser130, Tyr250, Gly151, Gln349
450432		0.54	-7.1	Tyr250, Gly151, Ser153, Ser130
6448		0.52	-7.6	Ser130, Ser153, Tyr250, Gly151
6057		0.65	-7.2	Ser153, Ser130, Tyr250, Phe249

TC, Tanimoto coefficient.

Huh-7 hepatocellular carcinoma cells, baclofen inhibited cell migration without affecting proliferation and Zhang *et al* (19) in breast cancer cell lines (4T1 and MCF-7), found that GABAergic signaling influenced metastasis through ERK1/2 phosphorylation, suggesting that a conserved mechanism exists across diverse cancer types.

The analysis of clonogenicity and cell survival in the present study revealed an effect of $GABA_B$ receptor activation on cancer cell behavior. Treating the cells with baclofen, a $GABA_B$ receptor agonist, caused significant inhibition of colony formation at concentrations as low as 50 μM, while preserving short-term cell viability even at a concentration of 200 μM. These results align with the observations made in the study by Zhang *et al* (19) regarding cell viability *in vitro*. Furthermore, the observed differential effect between acute survival and long-term proliferation implied a specific targeting of sustained growth pathways by baclofen rather than any immediate triggering of cell death mechanisms. This selective inhibition of metastatic behavior without direct cytotoxicity suggests baclofen could potentially serve as an anti-metastatic agent that specifically prevents cancer spread while minimizing adverse effects on healthy tissues. Such targeted anti-metastatic therapies represent an important clinical need, as metastasis is responsible for ~90% of cancer-related deaths (2). By inhibiting invasion pathways and EMT programming without inducing widespread cell death, GABA-B receptor agonists like baclofen might be particularly valuable in combination with conventional cytotoxic therapies or as maintenance treatment to prevent disease progression and metastatic dissemination in high-risk patients with minimal additional systemic toxicity.

At the molecular level, the modulation of EMT markers provides crucial mechanistic insights into how $GABA_B$ receptor activation mediates metastasis suppression (25). The plasticity of EMT is evident in its classification into three distinct types (I, II and III), each serving diverse roles in biological processes ranging from embryogenesis and organ development to wound healing and tumor metastasis (26). The increase in β-catenin expression that was observed in the present study, which occurred concurrently with a decrease in the levels of vimentin, indicated a transition toward a less metastatic phenotype. This molecular reprogramming was aligned with the functional observations made in the Transwell assay experiments showing reduced cell migration and invasion. Taken together, these findings corroborate those of the study by Gao *et al* (16), who reported that baclofen inhibits EMT in ovarian cancer cells, suggesting a direct regulation of EMT programming through $GABA_B$ receptor activation. The

mechanism likely involves GABA-B receptor-mediated modulation of key transcription factors that govern EMT. Upon GABA-B receptor activation, the resulting G protein-coupled signaling appears to stabilize epithelial phenotype markers like β-catenin while suppressing mesenchymal markers such as vimentin. This effect may be mediated through inhibition of transcriptional repressors of E-cadherin such as snail family transcriptional repressor 1, ZEB1 and twist related protein 1, which are known master regulators of the EMT process (7). As the present results showed that this occured independently of PI3K/Akt and MAPK pathways, this suggested that GABA-B receptors may influence EMT transcription factors through alternative signaling intermediates, possibly involving regulation of intracellular calcium levels or modifications to chromatin remodeling complexes that control EMT gene expression.

The PI3K/Akt signaling pathway is a notable intracellular signaling pathway that serves important roles in cell growth and cell proliferation. Abnormal activation of this pathway has been observed in numerous human cancers, including breast, lung, ovarian and prostate cancer (27). The present study revealed that the $GABA_B$ receptor-mediated anti-metastatic effects occurred independently of PI3K/Akt signaling in breast cancer cells, as evidenced by the unchanged phosphorylation levels. This finding stands in contrast with previous observations made in neuronal systems, where, in a previous study, baclofen was found to activate Akt through transactivation of the insulin-like growth factor 1 receptor (28). That the $GABA_B$ receptor-mediated anti-metastatic effects were found to occur independently of the PI3K/Akt pathway suggests the operation of tissue-specific signaling mechanisms, and that alternative pathways may exist to mediate the anti-metastatic effects. Alternative mechanisms could potentially be involved, including modulation of Rho GTPases controlling cytoskeletal dynamics, JAK/STAT signaling, calcium channel inhibition affecting adhesion, cAMP/PKA pathway influencing gene expression or Wnt/β-catenin signaling through GSK3β regulation.

The molecular docking analysis provided notable insights into potential natural $GABA_B$ receptor agonists. At present, baclofen [β-(4-chlorophenyl)] represents the only marketed $GABA_B$ receptor agonist (29), and it is primarily used as a muscle relaxant and antispastic agent (30). The identification of natural compounds with similar binding profiles to baclofen is particularly important, given the therapeutic potential of phytochemicals, which historically have been shown to exhibit reduced side effects compared with synthetic compounds (31). The three compounds that were identified in the present study to have enhanced hydrogen bonding profiles compared with baclofen warrant attention. Their interaction patterns with key receptor residues suggest potential agonist activity, which may provide new scaffolds for drug development. Both the favorable docking scores and specific hydrogen bonding interactions suggest that these compounds may serve as promising leads for developing novel $GABA_B$ receptor-targeted therapies.

Several key questions emerge from these findings. A primary limitation of the present study is the use of a single cell line (MDA-MB-231). However, the findings align with and extend previous research across multiple cancer models, and future validation in additional breast cancer cell lines such as SK-BR-3 (HER2-positive) and T-47D (hormone receptor-positive) would further strengthen these findings. Interestingly, the present findings appear to contrast with those reported by Gao *et al* (16), who demonstrated that baclofen, a $GABA_B$ receptor agonist, inhibited proliferation, migration, invasion and EMT in ovarian cancer cells by activating the $GABA_{B1}$ receptor. By contrast, the present results demonstrated that baclofen treatment promoted these processes in MDA-MB-231 breast cancer cells, while the antagonist CGP inhibited them. This apparent discrepancy with the present results may reflect tissue-specific differences in $GABA_B$ receptor signaling between ovarian and breast cancer cells. Similarly, Kanbara *et al* (17) reported that the $GABA_B$ receptor antagonist CGP had antitumor effects in chondrosarcoma cells, which aligns with the present observations in breast cancer. These conflicting findings underscore the complexity of GABAergic signaling in different cancer types and suggest that $GABA_B$ receptor modulation may elicit distinct, and sometimes opposing, effects depending on the specific cancer tissue context. This tissue-specificity in $GABA_B$ receptor signaling might be explained by differences in downstream signaling pathways or receptor coupling to various effector systems across different cell types. Future studies comparing $GABA_B$ receptor signaling mechanisms in these different cancer types would help elucidate the molecular basis for these differential responses. Additionally, the lack of *in vivo* experiments represents another notable limitation, as these findings need to be validated in a physiological context. Another important limitation was the use of single time points for each assay, which restricts the ability to fully distinguish between immediate and delayed effects of GABA-B receptor activation. This limitation particularly affects our understanding of the temporal dynamics of the observed anti-metastatic effects and whether they represent transient or sustained responses. Elucidating the complete signaling cascade that links $GABA_B$ receptor activation with EMT modulation also requires further study, as does identifying the potential impact on the tumor microenvironment. Furthermore, experimental validation of the computationally identified compounds represents a crucial next step in drug development.

In conclusion, baclofen, an agonist of the $GABA_B$ receptor, has been widely used for treating various diseases associated with GABA in previous years. Previous studies have demonstrated that baclofen exhibits marked regulatory effects in carcinoma (32,33). The present study has demonstrated that $GABA_B$ receptor activation functions as a selective regulator of breast cancer metastasis through multiple mechanisms. The present study has also demonstrated that baclofen treatment significantly inhibited the migratory, invasive and clonogenic properties of MDA-MB-231 cells through the modulation of EMT markers, as evidenced by the downregulation of vimentin and upregulation of β-catenin. These effects were found to occur independently of the PI3K/Akt and ERK1/2 signaling pathways, suggesting the existence of alternative signaling mechanisms. The present results may provide a future direction for the clinical treatment of breast cancer with $GABA_B$ receptor agonists and help to elucidate the role and viable mechanism of the $GABA_B$ receptor in breast cancer. In addition to these biological findings, the computational analysis identified three

promising natural compounds with potential $GABA_B$ receptor agonist activity. Collectively, these findings have extended current understanding of $GABA_B$ receptor function in breast cancer and offered additional mechanistic insights for targeted breast cancer treatments. These findings further validated and extended previous observations regarding $GABA_B$ receptor activation in breast cancer, demonstrating specific effects on metastatic potential in MDA-MB-231 cells.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Conceptualization, methodology, formal analysis and visualization were performed by MHA. Validation of experimental results was performed by ZYA, HHA, AA, NAA and ZTM through independent data verification, experiment replication, and critical assessment for reliability. Data collection was performed by MHA, ZYA, HHA, AA, NAA and ZTM. MHA wrote the original draft and ZYA, HHA, AA, NAA and ZTM revised and edited the manuscript. Project administration and funding acquisition were performed by MHA, NAA and ZTM. MHA and ZTM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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