Penfluridol: An Antipsychotic Agent Suppresses Metastatic Tumor Growth in Triple-Negative Breast Cancer by Inhibiting Integrin Signaling Axis

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Abstract

Metastasis of breast cancer, especially to the brain, is the major cause of mortality. The inability of anticancer agents to cross the blood-brain-barrier represents a critical challenge for successful treatment. In the current study, we investigated the antitumor potential of penfluridol, an antipsychotic drug frequently prescribed for schizophrenia with anticancer activity. We show that penfluridol induces apoptosis and reduces the survival of several metastatic triple-negative breast cancer (TNBC) cell lines. In addition, penfluridol treatment significantly reduced the expression of integrin α6, integrin β4, Fak, paxillin, Rac1/2/3, and ROCK1 in vitro. We further evaluated the efficacy of penfluridol in three different in vivo tumor models. We demonstrate that penfluridol administration to an orthotopic model of breast cancer suppressed tumor growth by 49%. On the other hand, penfluridol treatment inhibited the growth of metastatic brain tumors introduced by intracardiac or intracranial injection of breast cancer cells by 90% and 72%, respectively. Penfluridol-treated tumors from all three models exhibited reduced integrin β4 and increased apoptosis. Moreover, chronic administration of penfluridol failed to elicit significant toxic or behavioral side effects in mice. Taken together, our results indicate that penfluridol effectively reduces the growth of primary TNBC tumors and especially metastatic growth in the brain by inhibiting integrin signaling, and prompt further preclinical investigation into repurposing penfluridol for the treatment of metastatic TNBC.

Introduction

Breast cancer is the most diagnosed cancer and the second leading cause of mortality (1–4). Triple-negative breast cancer (TNBC) is characterized by the absence of receptors for estrogen (ER) and progesterone (PR) as well as lack of HER-2 gene (5). TNBC is considered to have basal characteristics, which are associated with more aggressive cancer phenotype (6). TNBC is more prevalent in younger females and accounts for about 10% to 20% of breast cancer incidence with poor prognosis (7). Metastatic TNBC is difficult to treat, thus leading to extremely poor survival. About 25% to 46% patients with TNBC are at higher risk of brain metastasis (8). Hence it is crucial to search new treatment options for patients with metastatic TNBC.

Integrins are heterodimeric adhesion family receptors, which facilitate communication of the cells with extracellular matrix and promote cell survival (9). Accumulating evidence suggests a major role of integrins in cancer. Integrin α6β4 has been implicated in breast cancer progression and metastasis, making it an attractive target for breast cancer therapy (10–13).

Published studies suggest an overall reduced risk of cancer in schizophrenic patients using neuroleptic agents (14, 15). Interestingly, few agents like chlorpromazine and thioridazine have shown anticancer activity (16–19). Penfluridol is an oral antipsychotic drug available since 1970 for the treatment of schizophrenia (20). Limited studies indicate that penfluridol exhibits anticancer activity; however, mechanism of the anticancer effect of penfluridol is not known (21).

In the present study, we investigated the antitumor potential of penfluridol in TNBC. We observed significant growth suppression of breast cancer cells by penfluridol treatment through inhibition of integrin signaling. Reduced cell migration and invasion of metastatic TNBC cells were also observed by penfluridol treatment. Oral administration of penfluridol suppressed the tumor growth and metastasis of breast cancer cells to brain by inhibiting integrin α6β4 signaling. To the best of our knowledge, this is the first report on the antitumor potential of penfluridol targeting integrin α6β4 signaling axis in TNBC.

Materials and Methods

Ethics statement

Investigation has been conducted in accordance with the ethical standards and according to approved protocol by Institutional Animal Care and Use Committee (IACUC).

Cell culture

Human triple-negative breast carcinoma cell lines MDA-MB-231 and 4T1 were purchased from ATCC and PerkinElmer, respectively. HCC1806 cells were kindly provided by Dr. Sophia Ran, Southern Illinois University-School of Medicine, Springfield, IL. The cell lines were maintained in DMEM supplemented...
Cytotoxicity studies

Cells were plated at a density of about 3,000 cells/well in 96-well plates and incubated overnight. The cells were then treated with different concentrations of penfluridol (Sigma-Aldrich). After desired duration of treatment (24, 48, and 72 hours), cells were fixed using ice cold 10% trichloroacetic acid followed by washing with water and staining with sulforhodamine B (SRB) dye. Plates were washed with 1% solution of acetic acid and the optical density was measured in 10 mmol/L Tris-base solution, using plate reader (BioTek Instruments) as described by us previously (22, 23).

Wound-healing assay

Wound-healing assay was performed as described by us earlier (24, 25). Briefly, 4T1 cells were incubated to form a monolayer in 6-well dishes. Wound was created by scratching the monolayer with a 1-mL sterile tip. The cells were washed with sterile PBS thrice to remove the floating cells and 4 μmol/L penfluridol was added to the cell culture media. The cells were fixed using 10% trichloroacetic acid at desired time points. The wound was imaged using bright field microscope (Olympus Inc.) after staining with SRB dye as described above. The wound widths were quantitated using ImageJ software.

Transwell cell invasion assay

Cell invasion was performed using Transwell Boyden’s chamber with 8.0 μm pore size membrane (BD Biosciences). The assay was performed according to manufacturer’s instructions and as described by us previously (24, 25). Briefly, serum-starved cells were seeded in the upper well of Boyden’s chamber. The lower chamber was filled with cell culture medium containing 10% FBS and VEGF as chemo-attractants. After 2 hours of incubation, 4 μmol/L penfluridol was added to upper chamber of the well. After 24 hours of penfluridol treatment, cells from the upper side of membrane were removed, whereas cells that migrated to the lower side of the membrane were fixed with 10% TCA and stained with 0.4% (w/v) SRB solution. The SRB dye was solubilized in 10 mmol/L Tris buffer and the absorbance was taken using a microplate reader (BioTek Instruments).

Integrin β4 and integrin α6 silencing

MDA-MB-231 cells were transfected with integrin β4 shRNA (Addgene) or integrin α6 siRNA (Cell Signaling Technologies) using Xfect (Takara Clonetech) or siPORT (Ambion Inc.) transfection reagent as per manufacturer’s protocol. Briefly, 2.5 μg shRNA plasmid was suspended in dilution buffer and Xfect polymer was added accordingly. The complexes were added to the cells after 10 minutes of incubation. In another experiment, cells were transfected with 100 nmol/L integrin α6 siRNA or scrambled siRNA using siPORT reagent and after 24 hours after transfection, cells were treated for additional 24 hours with 6 μmol/L penfluridol. The cells were collected after treatment and processed for Western blot analysis.

Laminin and TGFβ treatment

Laminin (Life Technologies) was diluted in PBS to attain a final concentration of 50 μg/mL and added to the wells (1 mL/well in a 6-well plate) or coverslips (500 μL/well in a 24-well plate) to cover the surface and incubated overnight at 4°C. Next day after removing laminin solution, wells were washed with PBS followed by a wash with prewarmed cell culture medium and the cells were plated. The cells were processed for Western blot analysis or microscopy after 24 hours treatment with penfluridol.

For TGFβ (Peprotech) treatment, cells were serum-starved overnight. Following day, media were replaced with normal cell culture media containing 10% serum and 20 ng/mL TGFβ 1 hour before penfluridol treatment. After 24-hour treatment with 6 μmol/L penfluridol, cells were collected and processed for Western blot analysis.

Western blot analysis

The whole-cell lysates were prepared using 4% (w/v) CHAPS in urea-tris buffer. Proteins from whole-cell lysates were subjected to SDS-PAGE and the resolved proteins were transferred to PVDF membrane. The membranes were probed for primary antibodies against integrin α6, integrin β4, integrin α4, integrin αv, integrin β1, integrin β3, p-FAK (Y397), FAK, p-paxillin (Y118), paxillin, Rac1/2/3, p-Rac1 (S71), ROCK1, and cleaved caspase-3. All primary antibodies were purchased from Cell Signaling Technologies except integrin β4 and FAK (Santa Cruz Biotechnology, Inc.). The membranes were developed as described by us previously (22, 23, 26, 27).

Immunoprecipitation

Immunoprecipitation was performed as described by us previously (10, 28–30). Briefly, 0.75 × 10^6 MDA-MB-231 cells were plated in 100-mm tissue culture dishes and treated with 6 μmol/L penfluridol. After 24-hour penfluridol treatment, whole-cell lysates were prepared using RIPA buffer and immunoprecipitated with integrin β4 antibody (Santa Cruz Biotechnology, Inc.). Immune complexes were resolved on SDS-PAGE and immunoblotted for integrin α6.

PCR

Total RNA was extracted from control and penfluridol-treated cells using TRIzol reagent (Life Technologies, Inc.) according to manufacturer instructions and the cDNA synthesis was carried out as described by us earlier (29). About 2 μg of template cDNA was used for cycling in a thermal cycler (Thermo Fisher). The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized under UV light using BioRad Versa Doc imager.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described by us earlier (26). MDA-MB-231 cells were plated in a 24-well plate on a coverslip (uncoated or laminin coated) at a density of 0.1 × 10^6 cells/well and allowed to attach overnight followed by treatment with 4 μmol/L penfluridol for additional 24 hours. The cells were fixed with formalin and permeabilized using Triton-X100 solution. After blocking with goat serum, cells were incubated overnight with primary antibody for integrin β4 (1:150). Next day, cells were washed and incubated with AlexaFluor 488 secondary antibody (Invitrogen). After washing, the coverslips were mounted on slides using Prolong Gold Antifade Reagent with DAPI (Life Technologies) and images were taken using fluorescence microscope (Olympus).
Figure 1.
Penfluridol suppresses cell survival, cells migration, and invasion. A, MDA-MB-231, HCC1806, and 4T1 cells were treated with different concentrations of penfluridol for 24, 48, and 72 hours. Cell survival was measured by sulforhodamine B assay to estimate IC50 values. The experiments were repeated three times with 8 replicates in each experiment. B, 4T1 cells were grown in 6-well plates to form monolayer. Wounds were created using 1 mL sterile tip. Cell migration capacity was measured by the time taken to heal the wound in control and penfluridol (4 μmol/L)-treated cells. Statistically different at P < 0.05 when compared with control. C, 4T1 cells were starved in serum-free medium overnight before plating cells in Transwell. Invading potential of the cells was estimated by staining the cells invaded to lower side of the membrane using sulphorhodamine B dye. Invading potential of the cells in the treatment was compared with the control. Values were plotted as mean ± SD. Experiment was repeated three times and considered statistically significant with control at P < 0.05.
Breast tumor orthotopic model

Female Balb/c mice (4–6 weeks old) were obtained from Harlan Laboratories. The experiments were conducted in strict compliance with the regulations of IACUC, Texas Tech University Health Sciences Center (Amarillo, TX). Exponentially growing 4T-1 cells were harvested, washed twice with PBS, and resuspended in PBS at a density of $0.7 \times 10^6$ cells per mL. A suspension of 0.1 mL containing $0.07 \times 10^6$ cells was injected in the inguinal mammary fat pads of each recipient mouse. Tumor volumes were measured three times a week as described by us previously (10, 31) and calculated using the formula $\text{length} \times \text{width}^2/2$; refs. 32, 33. Two days after tumor cells injection, mice were randomly segregated into two groups with seven mice in each group. Test group of mice received 10 mg/kg penfluridol by oral gavage every day till day 27, whereas control mice received vehicle alone. Penfluridol stock was made in DMSO, which was further diluted in water/PEG300/ethanol/2% acetic acid in 8:3:1 v/v (34). Experiment was terminated at day 27 by euthanizing mice with CO2 overdose. The tumors were removed aseptically from each mouse, weighed, and snap frozen in liquid-nitrogen for Western blot analysis. A part of tumor was fixed in formalin for IHC analysis.

Intracardiac brain metastasis model

Female Balb/c mice (4–6 weeks old) were obtained from Charles River and maintained as per the IACUC guidelines. For the metastatic breast cancer model, we used a method first described by Conley and colleagues in 1979 (35) and since then it is widely used to study metastasis to bone and brain (36, 37). Briefly, 4T1 cells expressing luciferase (PerkinElmer), were
Penfluridol induces apoptosis through suppression of integrin α6β4 signaling cascade. MDA-MB-231 cells were treated with penfluridol (6 μmol/L) for 24 hours after transfecting the cells with integrin α6 siRNA (A) and transfecting the cells with integrin β4 shRNA (B). C, cells were plated in laminin-coated plate followed by treatment with 6 μmol/L penfluridol for 24 hours. D, cells were pretreated with 20 ng/mL TGFβ for one hour followed by treatment with penfluridol as described above. Levels of p-FAK, p-paxillin, p-Rac1, Rac1/2/3, ROCK1, cleaved caspase-3 were evaluated by Western blotting. Figure shown is the representative blot of at least three independent experiments. Blots were quantitated and normalized with actin. E, integrin β4 was immunoprecipitated from control and 6 μmol/L penfluridol-treated MDA-MB-231 cells and probed for integrin α6. F, mRNA level of integrin α6 was evaluated after treating MDA-MB-231 cells with 6 μmol/L penfluridol for 24 hours by RT-PCR. G, nuclear localization of integrin β4 by fluorescence microscopy in MDA-MB-231 cells treated with 4 μmol/L penfluridol for 24 hours. Green fluorescence, integrin β4; blue, DAPI.
harvested, washed and resuspended in sterile PBS at a density of $2.5 \times 10^4$ cells/50 μL. Mice were injected with 50 μL of the cell suspension into the heart’s left ventricle using stereotaxic apparatus. Tumor growth in mice was monitored by using non-invasive imaging technique (IVIS, PerkinElmer). After 3 weeks, mice were sacrificed and brain was collected aseptically to collect 4T1-luc cells. The 4T1-luc cells were selected using 60 μmol/L 6-thioguanine and cultured further and named them brain seeking 4T1-luc cells. These cells ($2.5 \times 10^4$ cells/50 μL) were reinjected into the left ventricle of a new set of mice under isofluorane anesthesia. Animals were monitored till they became conscious. The mice injected with 4T1-luc cells were randomized and divided into two groups with 5 mice in each group. The 4T1 breast tumor cells usually reach the brain within 5 minutes following intracardiac injection as observed by imaging. The treatment group

Figure 4.
Penfluridol suppresses the growth of breast tumor and integrin signaling in vivo. A, about $0.07 \times 10^6$ 4T1 breast cancer cells were injected orthotopically in the mammary fat pads of 4 to 6 weeks old Balb/c female mice. Treatment with 10 mg/kg penfluridol by oral gavage everyday started 2 days after tumor cells injection till day 27. Values were plotted as mean ± SEM. B, tumors were weighed once isolated from control- and penfluridol-treated mice. Values were plotted as mean ± SEM. C, representative tumor pictures from control and penfluridol-treated mice. Orthotopically implanted tumors were removed aseptically after terminating the experiments. Tumors were homogenized, lysed, and analyzed for integrin α6, integrin β4, FAK, ROCKI, Rac1/2/3, and cleaved caspase-3. Actin was used as loading control. D, each lane of blot represents tumor from individual mouse. E, blots were quantitated, normalized with actin, and represented as bars. Values were plotted as mean ± SEM. F, tumors were sectioned and immunostained for integrin β4 and cleaved caspase-3, as described in Materials and Methods.

Figure 5.
Penfluridol inhibits the growth of metastatic breast tumors in the brain in intracardiac model. About $2.5 \times 10^4$ 4T1-luc brain-seeking aggressive breast cancer cells were injected in left ventricle of 4 to 6 weeks old female Balb/c mouse heart. Mice were treated with 10 mg/kg penfluridol by oral gavage everyday till day 12. A, brain luminescence (photons/sec of each mouse was normalized with initial average luminescence of all the control and treated group mice. Relative increase in brain luminescence of control and treated group was plotted against days till day 12. B, after terminating the experiment, brain of each mouse was imaged and luminescence in respective group was plotted. Values were plotted as mean ± SEM. Representative brains from control and penfluridol-treated mice. C, brains were processed, sectioned, and immunostained for integrin β4 and cleaved caspase-3.
received 10 mg/kg penfluridol by oral gavage starting the same day after cell injection, whereas control group received vehicle only. The mice were humanely sacrificed at day 12 as the control mice started showing signs of sickness due to metastatic tumor burden in brain. The brains were collected, imaged for luminescence, and fixed in formalin overnight and processed for IHC analysis.

Intracranial tumor model

Female Balb/c mice (4–6 weeks old) from Harlan Laboratories were used for intracranial injection and the experiments were conducted in strict compliance with the regulations of IACUC, Texas Tech University Health Sciences Center. Exponentially growing 4T1-luc cells were harvested, washed twice with sterile PBS, and resuspended in PBS at a density of $5 \times 10^6$ cells per mL. A suspension of 5 mL containing $0.025 \times 10^6$ cells were injected by intracranial route at a flow rate of 1 mL/minute using Quintessential Stereotaxic Injector (Stoelting Co.) in each recipient mouse using stereotaxic apparatus. Following this, mice were randomly divided into two groups with 6 mice in each group. Of note, 10 mg/kg penfluridol by oral gavage was given to each mouse everyday till day 21. Experiment was terminated by humanely euthanizing the mice with CO2 overdose and mice brain were carefully dissected out, weighed, imaged for luminescence, and processed for IHC staining.

Immunostaining of tissue sections

The IHC was performed as previously described by us (10, 38). Briefly, fixed brains were dehydrated, embedded in paraffin, and sectioned into 5 μm thick sections using microtome (Leica Microsystems Inc.). The sections were deparaffinized and rehydrated using xylene, ethanol, and double-distilled water washes. Antigens were unmasked by boiling the sections in 10 mmol/L sodium citrate buffer (pH 6.0) and the sections were washed and incubated in 3% hydrogen peroxide solution. The sections were blocked with 5% goat
serum and incubated with primary antibodies for integrin β4 (1:100), and cleaved caspase-3 (1:100) overnight at 4°C. Next day the slides were stained using Ultravision ONE HRP Polymer Kit (Thermo Fisher Scientific) as per the manufacturer’s instructions. The sections were counterstained with Mayer’s hematoxylin and dehydrated. The slides were mounted using Permount (Fisher Scientific) and imaged using Olympus microscope (Olympus America Inc.).

Dose tolerance and mice behavioral analysis

Female CD1 mice (4–6 weeks old) were obtained from Charles River. The use of CD1 mice and their treatment was approved by the IACUC, Texas Tech University Health Sciences Center. Mice were randomly divided into two groups with 5 mice per group. Mice were administered 10 mg/kg penfluridol by oral gavage every day for 55 days. Control mice received the vehicle only. Mice weights were monitored once a week and mice were observed for general signs of toxicity. After 55 days of treatment, behavioral activity of mice was assessed using Versamax (AccuScan Instruments Inc.). Versamax is a ventilated chamber equipped with infrared sensors along the side wall to monitor mouse activity. Each mouse was acclimatized in chamber for 15 minutes before taking the reading. The readings were taken for control and penfluridol treatment groups. At the end of experiment, mice were humanely euthanized and plasma was carefully collected for analysis of liver transaminases. The enzymatic activities of AST and ALT were determined using a commercially available kit (Pointe Scientific, Inc.), according to manufacturer’s instructions and as described previously (10). Mice organs were also collected and weighed for comparison between control and penfluridol treatment group.

Statistical analysis

Prism 6.0 software was used for all the statistical analysis (GraphPad Software Inc.). Results are represented as mean ± SD or SEM. Statistical significance was analyzed using Student t test or Mann–Whitney test and outcomes were considered statistically significant at P < 0.05.

Results

Penfluridol suppresses proliferation of TNBC breast cancer cells

To evaluate the growth-suppressive effects of penfluridol, we first performed the cytotoxicity assay in MDA-MB-231, HCC1806, and 4T1 TNBC cells. The cells were treated with varying concentrations of penfluridol for 24, 48, and 72 hours. Our results showed that treatment with increasing concentrations of penfluridol significantly suppressed the growth of all the three breast cancer cell lines in a concentration and time-dependent manner. The IC_{50} of penfluridol after 24 hours treatment ranged 6 to 8 μmol/L in all the three cell lines (Fig. 1A). The IC_{50} values were further reduced to about 4 to 5 μmol/L and to 2 to 4 μmol/L after 48 and 72 hours treatment, respectively, in all the cell lines (Fig. 1A). These results suggest potential cytotoxic effects of penfluridol in TNBC cells.

Penfluridol inhibits migration and invasion of breast cancer cells

To determine the effects of penfluridol on metastatic potential of cells, cell migration using wound-healing assay was evaluated. Our results indicated that the migration of penfluridol-treated 4T1 cells was significantly delayed as compared with control cells. Penfluridol treatment inhibited the migration of 4T1 cells by 61% and 76% at 18 and 36 hours, respectively (Fig. 1B). Furthermore, the effect of penfluridol on cell invasion was confirmed by Transwell invasion assay. Our results showed that invasion of penfluridol-treated cells was only 60% as compared with 100% in control cells (Fig. 1C). These observations indicated that penfluridol treatment inhibits cell migration and invasion of breast cancer cells, suggesting antimetastatic potential.

Penfluridol inhibits integrin signaling

To elucidate the molecular mechanism of the growth-suppressive effects of penfluridol, we performed Western blot analysis of whole-cell lysates of MDA-MB-231, HCC1806, and 4T1 cells treated with 0, 2.5, 5, and 7.5 μmol/L penfluridol for 24 hours. Our results showed that expression of integrin α6 and integrin β4 was significantly reduced in a concentration-dependent manner by penfluridol treatment in MDA-MB-231 and HCC1806, human breast cancer cell lines (Fig. 2A and B). Surprisingly, in 4T1, a murine breast cancer cells, we did not observe much change in integrin α6 but integrin β4 expression was significantly reduced in a concentration-dependent manner by penfluridol treatment (Fig. 2C). Penfluridol treatment also reduced the expression of integrin α4, integrin αv, integrin β1 and integrin β3 in concentration-dependent manner in MDA-MB-231, HCC-1806, and 4T1 cells (Supplementary Fig. S1). In addition, we observed a notable inhibition of the downstream effector molecules of integrin signaling such as FAK, p-axillin (Y118), and axillin by penfluridol treatment. Integrin signaling modulates Rac and ROCK1 proteins to activate cell migration and invasion (Fig. 2A and B). Interestingly, penfluridol treatment significantly inhibited p-Rac1 (S71), Rac1/2/3, and ROCK1 expression (Fig. 2A and B). These results indicate that penfluridol suppresses cell survival and motility by inhibiting integrin signaling.

Silencing integrins α6 and β4 enhances the effects of penfluridol

To confirm the role of integrins in penfluridol attributed effects, we genetically silenced integrin α6 and β4 in MDA-MB-231 cells using siRNA and shRNA, respectively. Using siRNA, silencing 32% integrin α6 caused suppression of p-FAK (Y397), p-Rac-1, ROCK1 (Fig. 3A), and augmented the effects of penfluridol treatment on integrin signaling as well as cleavage of caspase-3 (Fig. 3A). Similarly, using shRNA, we were able to inhibit about 43% integrin β4 expression (Fig. 3B). Our results further showed that silencing of integrin β4 significantly suppressed the levels of p-FAK (Y397), Rac1/2/3 as well as ROCK1 (Fig. 3B). The effect of penfluridol in suppressing the level of these molecules was further enhanced in MDA-MB-231 cells with silenced integrin β4. A 17-fold increased cleavage of caspase-3 was observed in penfluridol-treated cells with silenced integrin β4 expression as compared with only 6-fold in the cells with basal expression of integrin β4 (Fig. 3B). These observations prove that penfluridol treatment inhibits integrin α6/β4 signaling to suppress breast cancer cell growth as well as migration and invasion potential.
Activation of integrins α6 and β4 signaling suppresses efficacy of penfluridol

We further used laminin, the ligand of integrin α6β4 and TGFβ, to activate integrin signaling and then evaluated the effects of penfluridol in breast cancer cells. Our results showed that the cells cultured on laminin-coated surface increased p-FAK(Y397) and p-paxillin (Y118) by 1.10- and 1.70-fold, respectively; however, levels of these phosphorylated protein was significantly reduced by penfluridol treatment (Fig. 3C). In addition, increased integrin α6β4 signaling by laminin reduced cleavage of caspase-3 induced by penfluridol treatment in MDA-MB-231 cells (Fig. 3C). Similarly, TGFβ treatment also activated integrin signaling, as exhibited by increased phosphorylation of FAK (Y397) and p-paxillin (Y118, Fig. 3D). In line with these observations, activation of integrin α6β4 signaling by TGFβ blocked the cleavage of caspase-3 mediated by penfluridol in MDA-MB-231 cells (Fig. 3D). Taken together these results confirmed integrin α6β4 as targets of penfluridol in suppressing the growth of breast cancer cells.

Penfluridol treatment disrupts integrin α6β4 heterodimer

To delineate the mechanism of inhibition of integrins, effects of penfluridol on heterodimerization of integrin α6 with integrin β4 were evaluated by immunoprecipitation studies. Immunoprecipitated integrin β4 from penfluridol-treated MDA-MB-231 cells showed reduced association of integrin α6 with integrin β4 (Fig. 3E). We further evaluated the effect of penfluridol on integrin α6 at the transcriptional level by performing PCR analysis. Our results showed that penfluridol treatment inhibited the mRNA levels of integrin α6 by 76% in MDA-MB-231 cells (Fig. 3F). In addition, the immunofluorescence analysis also confirmed inhibition of integrin β4 by penfluridol treatment (Fig. 3G). We also induced integrin β4 signaling by using laminin and then treated the cells with penfluridol. The green staining for integrin β4 was reduced significantly in the cells treated with penfluridol or in cells treated with laminin and penfluridol combination. Taken together, these results demonstrate that integrin α6β4 are the targets of penfluridol in breast cancer cells.

Penfluridol inhibits the growth of 4T1 orthotopic tumors

To test the efficacy of penfluridol in vivo, we implanted aggressive 4T1 murine breast tumor cells, representing stage IV breast cancer, orthotopically in the mammary fat pads of Balb/c mice and the mice were treated with 10 mg/kg penfluridol by oral gavage every day. Our results showed significant suppression of tumor growth in penfluridol-treated mice as compared with control mice. At the end of experiment, average tumor volume of penfluridol-treated group was about 342 mm³ as compared with 668 mm³ of control group, indicating 49% reduction in tumor volume (Fig. 4A). Tumors were collected and weighed after humanely euthanizing the mice. Average weight of penfluridol-treated tumors was about 42% less as compared with control group (Fig. 4B and C). Tumor lyses were subjected to Western blot analysis. In Fig. 4D, each band represents lystate from a separate tumor. In agreement with our in vivo observation with 4T1 cells, no significant change in the expression of integrin α6 was observed in penfluridol-treated tumors as compared with control tumors (Fig. 4D and E). However, a remarkable suppression of FAK, ROCK1, Rac1/2/3, and enhanced cleavage of caspase-3 was observed in penfluridol-treated tumor lystate (Fig. 4D and E). These observations were also confirmed by IHC staining of tumors from control and penfluridol-treated mice for integrin β4 and cleaved caspase-3. Our results demonstrated reduced expression of integrin β4 as well as increased cleavage of caspase-3 in tumor samples from penfluridol-treated mice (Fig. 4F). These results indicated that breast tumor growth suppression by penfluridol was associated with inhibition of integrin signaling and induction of apoptosis.

Penfluridol inhibits in vivo brain metastasis of 4T1 cells

The in vivo efficacy of penfluridol was further validated in an in situ metastatic breast cancer model. The 4T1-luc cells were injected into the left ventricle of mouse heart so that the cells lodge in the brain. Luminescence was detected in the brain of mice within minutes of intra-cardiac injection of the tumor cells (data not shown). Mice in experimental group received 10 mg/kg penfluridol by oral gavage every day. Control mice bearing metastatic breast tumor in brain starts succumbing after 12 days so the experiment was terminated at day 12. Our results showed an increase in brain luminescence starting day 6 after injection (Fig. 5A). At the end of the experiment, there was a massive increase in luminescence signal in control group as compared with penfluridol-treated mice (Fig. 5A). On the basis of luminescence, our results showed about 90% inhibition in tumor growth by penfluridol treatment (Fig. 5A). At the end of the experiment, brain from control- and penfluridol-treated mice was removed and imaged. Average brain luminescence from penfluridol-treated group was also suppressed by 90% as compared with control group (Fig. 5B). The brain from control and penfluridol-treated mice was analyzed by IHC. Consistent with our previous observations, tumors from penfluridol-treated mice exhibited reduced expression of integrin β4 and increased staining for cleaved caspase-3 (Fig. 5C). These results clearly indicated that penfluridol treatment suppressed metastatic growth of TNBC cells by inhibiting integrins and inducing apoptosis.

Penfluridol inhibits the growth of 4T1 tumors in intracranial tumor model

The antitumor effect of penfluridol was further validated in an intracranial tumor model. 4T1-luc tumor cells were injected...
directly into the brain through intracranial injection using stereotaxic apparatus and the mice were treated with 10 mg/kg penfluridol by oral gavage every day. Our results showed a steady increase in brain luminescence, indicating fast tumor growth of 4T1-luc cells in the brain of control mice, whereas not much increase of luminescence was observed in the brain of penfluridol-treated mice. At the end of the experiment (day 21), luminescence in control mice was 3.5-fold higher than the luminescence in penfluridol-treated mice (Fig. 6A). We also analyzed luminescence in the isolated brains from both groups after euthanizing the mice. Our results showed a clear suppression of tumor growth by penfluridol treatment as indicated by reduced luminescence in penfluridol-treated brains as compared with control brains (Fig. 6B). In addition, we also observed reduction in average brain weight of penfluridol-treated group, suggesting reduced tumor load (Fig. 6C). The isolated brains were also examined by IHC. Consistently, we observed suppression of integrin β4 expression as well as increase in cleaved caspase-3 in the tumors from the brain of penfluridol-treated mice as compared with control mice (Fig. 6D). These results established the growth inhibitory effects of penfluridol in metastatic tumors.

**Penfluridol does not exhibit any toxicity in vivo in a chronic toxicity model**

Our *in vitro* and *in vivo* studies conclusively showed breast tumor growth suppression by penfluridol in three different *in vivo* models. However, it was not clear whether long-term treatment with penfluridol would cause any unwanted side effects or toxicity. Hence, we studied long-term effects by treating the mice with 10 mg/kg penfluridol by oral gavage every day for 55 days. General signs of toxicity, such as, body weights, organ weights, and plasma transaminases (ALT, AST) were evaluated. Our results showed no significant difference in the overall weights of penfluridol-treated mice as compared with control mice (Fig. 7A). Penfluridol treatment modestly increased ALT activity, whereas AST activity was reduced. The overall high basal level of AST and ALT in our study compared with reported normal range could be due to lysis of RBC while collection of plasma (Fig. 7B and C). We also weighed the brains, livers, kidneys, and spleens of control and penfluridol-treated mice after the experiment was terminated. No difference in the average weights of any of these critical organs was observed in penfluridol-treated group as compared with control group of mice (Fig. 7D–G). These results suggested that chronic treatment with 10 mg/kg penfluridol was not associated with any toxicity or side effects.

**No apparent effects of penfluridol on behavioral activity of mice**

Penfluridol is a CNS acting drug; hence besides general signs of toxicity, we also monitored behavioral activity of mice after long-term penfluridol administration. The behavioral activity of mice was monitored after 55 days of penfluridol treatment using Versamax (AccuScan Instruments Inc.). The readings were taken after administration of 10 mg/kg penfluridol every day for 55 days. Our results showed no significant changes in terms of clockwise or counter-clockwise movement, total distance covered, vertical and horizontal activity in penfluridol-treated mice as compared with control mice (Fig. 7H–L). These results suggested that chronic administration of penfluridol does not have any significant effect on the behavioral activity of mice, indicating that perhaps it is relatively safe for long term use.

**Discussion**

Our current study provides the first *in vitro* and *in vivo* evidence for significant activity of an antipsychotic drug penfluridol against metastatic TNBC, a type of breast cancer, which is currently untreatable. Our results indicated that penfluridol treatment reduced the proliferation of TNBC by inhibiting integrin α6β4 signaling axis. Surprisingly, inhibition of integrin α6 was not observed by penfluridol treatment in 4T1, a murine breast cancer cell line. Inhibitory activity of penfluridol on integrin α6 axis was also confirmed by silencing integrin α6 and integrin β4, which further enhanced the effects of penfluridol in TNBC cells. Furthermore, activation of integrin α6β4 by laminin or TGFβ, suppressed the inhibitory effect of penfluridol, which was demonstrated by reduction in cleavage of caspase-3. To determine the efficacy of penfluridol *in vivo*, we used three different tumor models. Interestingly, penfluridol suppressed the growth of breast tumors in brain as well as in the breast as evaluated in intracardiac, intracranial, and orthotopic models respectively. Suppression of metastatic breast tumors in brain and primary breast tumors by penfluridol was due to inhibition of integrin signaling as demonstrated by Western blot analysis and IHC staining of tumors and consistent with our *in vitro* observations. Moreover, antitumor dose of penfluridol used in our experiment was safe to use without any side effect or behavioral changes as shown in our chronic toxicity study where 10 mg/kg penfluridol was administered by oral gavage in mice everyday till 55 days. Hence our study reveals a highly impressive activity of penfluridol against TNBC.

Published studies indicate reduced cancer incidence with consumption of neuroleptic agents in patients with schizophrenia (14, 15, 39). Penfluridol is an approved antipsychotic drug for schizophrenia and upon administration shows good bioavailability in brain. Few studies have suggested that antipsychotic drugs such as haloperidol, perphenazine, and fluphenazine have anticancer effects (17, 40). Consistent with those studies, our results demonstrated the antitumor activity of penfluridol in breast cancer model.

Wu and colleagues recently have shown the anticancer effects of penfluridol by modulating cholesterol homeostasis (21). The study showed that IC50 of penfluridol was between 2–4 μmol/L after 48-hour treatment in B16/F10, LL/2, 4T1, and CT26 cells. Similar to Wu and colleagues, we observed an IC50 of about 3.7 μmol/L in 4T1 cells at 48 hours. Our results showed an IC50 ranging between 5–8 μmol/L and 3–5 μmol/L after 24 and 48-hour treatment, respectively, in all the breast cancer cell lines tested. In another recent study, penfluridol was shown to induce apoptosis in pancreatic cancer cells by activating protein phosphatase 2A (PP2A; ref. 41). Our results also showed suppression of cell migration and invasion by penfluridol treatment, suggesting antimetastatic effects in TNBC cells.

Integrins have recently gained attention as an important therapeutic target in various cancer types (42, 43). The heterodimerization of integrin α6 with β4 plays significant role in breast tumor progression. In fact, overexpression of integrin α6β4 has been observed in breast tumors (10). Our results showed significant suppression of integrin α6β4 by penfluridol treatment. Integrin signaling is known to be mediated by downstream activation of FAK, paxillin, Rac, ROCK proteins (44). Interestingly, penfluridol treatment also inhibited the expression as well as activation of these downstream proteins.
In all the breast cancer cell lines tested. We have previously shown that TGFβ treatment increased the expression of integrin α6, leading to reduced cleavage of caspase-3 induced by curcumin B (10). In agreement, results from current study also showed increased integrin α6 expression by TGFβ treatment and reduced cleavage of caspase-3 by penfluridol, indicating integrin α6β4 as a target of penfluridol in breast cancer cells. Integrin α6β4 has been shown to have role in cell migration by laminin (45). Hence laminin-coated culture dishes were used to evaluate the anticancer effects of penfluridol. Our results showed that laminin reduced penfluridol-mediated suppression of integrin signaling. Interestingly, we also observed disruption of integrin α6β4 dimerization by penfluridol treatment. Furthermore, our results showed a significantly reduced mRNA level of integrin α6 by penfluridol treatment, indicating that the inhibition of integrin α6 was at transcriptional level. In addition to integrin α6β4, integrin αvβ3 heterodimer plays a critical role in breast cancer metastasis to bones. Our results showed that penfluridol treatment downregulated the expression of ov and β3 integrin as well. These results clearly indicate that penfluridol specifically inhibits integrin signaling in TNBC cells.

Penfluridol is an orally bioavailable antipsychotic drug with doses ranging from 20 to 250 mg/week. Wu and colleagues did not observe any statistically significant A1 tumor growth suppression with an oral dose of 0.06 to 0.12 mg/week/mouse (3–6 mg/kg/week) penfluridol in mice (21). Interestingly, our results showed significant tumor growth suppression in all the three in vivo models by 10 mg/kg penfluridol administration every day by oral gavage. The human equivalent dose of 10 mg/kg penfluridol administered in mice is about 0.83 mg/kg. Moreover, 10 mg/kg everyday administration of penfluridol in mice is equivalent to 1.4 mg/week. Penfluridol treatment substantially reduced the establishment of metastasized breast tumors in the brain of mice in the intracardiac model. Penfluridol treatment also significantly inhibited the growth of established breast tumors in the brain of mice in an intracranial model. However, the inhibitory effect of penfluridol was not as robust in orthotopic tumor model as compared with intracardiac or intracranial model. The exact reason behind the difference in efficacy of penfluridol in different models is not clear at this point. Consistent with our in vitro observations, the tumor growth-suppressive effects of penfluridol were associated with reduced expression of integrin β4 and increase in cleavage of caspase-3, as evaluated by HIC in all the three tumor models, suggesting similar mechanism. Mice did not show any significant behavioral side effects or any sign of toxicity when treated with 10 mg/kg penfluridol by oral gavage for 55 days.

Overall, our study provides convincing results to establish strong antitumor and antitumorigenic effects of penfluridol in TNBC. To the best of our knowledge, ours is the first study to demonstrate use of penfluridol for metastatic TNBC. Taken together, the outcomes from our study are very encouraging as they lay a foundation for repurposing penfluridol for TNBC, which currently lacks any specific treatment options.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Ranjan, P. Gupta, S.K. Srivastava

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Ranjan, P. Gupta, S.K. Srivastava

Writing, review, and/or revision of the manuscript: A. Ranjan, P. Gupta, S.K. Srivastava

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.K. Srivastava

Study supervision: S.K. Srivastava

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