Anti-tumor Effects of Penfluridol through Dysregulation of Cholesterol Homeostasis

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Abstract

Background: Psychiatric patients appear to be at lower risk of cancer. Some antipsychotic drugs might have inhibitory effects on tumor growth, including penfluridol, a strong agent. To test this, we conducted a study to determine whether penfluridol exerts cytotoxic effects on tumor cells and, if so, to explore its anti-tumor mechanisms.

Methods: Growth inhibition of mouse cancer cell lines by penfluridol was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cytotoxic activity was determined by clonogenic cell survival and trypan blue assays. Animal tumor models of these cancer cells were established and to evaluate penfluridol for its anti-tumor efficacy in vivo. Unesterified cholesterol in cancer cells was examined by filipin staining. Serum total cholesterol and tumor total cholesterol were detected using the cholesterol oxidase/p-aminophenazone (CHOD-PAP) method.

Results: Penfluridol inhibited the proliferation of B16 melanoma (B16/F10), LL/2 lung carcinoma (LL/2), CT26 colon carcinoma (CT26) and 4T1 breast cancer (4T1) cells in vitro. In vivo penfluridol was particularly effective at inhibiting LL/2 lung tumor growth, and obviously prolonged the survival time of mice bearing LL/2 lung tumors implanted subcutaneously. Accumulated unesterified cholesterol was found in all of the cancer cells treated with penfluridol, and this effect was most evident in LL/2, 4T1 and CT26 cells. No significant difference in serum cholesterol levels was found between the normal saline-treated mice and the penfluridol-treated mice. However, a dose-dependent decrease of total cholesterol in tumor tissues was observed in penfluridol-treated mice, which was most evident in B16/F10-, LL/2-, and 4T1-tumor-bearing mice.

Conclusion: Our results suggested that penfluridol is not only cytotoxic to cancer cells in vitro but can also inhibit tumor growth in vivo. Dysregulation of cholesterol homeostasis by penfluridol may be involved in its anti-tumor mechanisms.

Keywords: Antipsychotics drugs - penfluridol - anti-tumor effect - cholesterol
increase abnormally in the human neuroblastoma cell treated with pimozide or olanzapine (Wiklund et al., 2010). Despite the above findings, the exact mechanisms leading to the anti-tumor effect of APDs are unknown and the majority of these observations are in vitro studies. The question remained as to whether APDs would still have an anti-tumor effect in vivo. Further research was needed to explore the anti-tumor effect and elucidate the potential mechanisms.

Penfluridol (C28H27ClF5NO), an antipsychotic drug similar to pimozide, is commonly used in clinical settings. It has been reported that pimozide has a stronger inhibitory effect on cancer cells than other types of APDs, so we speculated that penfluridol should demonstrate an equally powerful anti-tumor effect. To this end, we conducted a study to determine whether penfluridol exerts a cytotoxic action against tumor cells, and, if so, to examine the possible mechanism(s) of this effect and outline the potential implications for the treatment of cancer by penfluridol.

Materials and Methods

Materials

In our study, we used the following materials: 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO); dimethyl sulfoxide (DMSO; Sigma, St Louis, MO); a protein assay kit (Trypan blue staining cell viability assay kit; Beyotime, Shanghai, China); crystal violet (Beyotime, Shanghai, China); ECL detection system (Millipore, Billerica, MA); Filipin staining kit (Genmed Co. Ltd, Shanghai, China); and penfluridol (purity>98%; ZhiQi Pharmaceutical Co. Ltd, Wuhan, China). The penfluridol was prepared in ethanol at a concentration of 10 mmol/L. Drug stock was diluted in RPMI 1640 containing 10% FBS (Life Technologies, Bedford, MA) when required for assays.

Tumor cell line and culture

Murine B16 melanoma (B16), LL/2 lung carcinoma (LL/2), CT26 colon carcinoma (CT26) and 4T1 breast cancer (4T1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 (Life Technologies, Bedford, MA) containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin in a humid chamber at 37°C, 5% CO₂ in atmosphere.

Cell viability assay

The cell viability of penfluridol-treated cancer cells was determined by the MTT assay. Briefly, cells (4-5x10⁴) were seeded in 96-well plates and cultured for 24 h, followed by the addition of different concentrations of penfluridol 0 (vehicle) - 10 μmol/L treatment for 24 h, 48 h and 72 h. A volume of 10 μl of 10 mg/ml MTT was added per well and incubated for 4 h at 37°C, then the supernatant fluid was removed and 150 μl of DMSO was added for 15 min. The light absorptions (OD, optical densities) were measured at 570 nm with a SpectraMaxM5 Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). All experiments were performed in triplicate. The effect of penfluridol on tumor cell viabiliy was expressed by IC50 of each cell line. The effect of penfluridol on the proliferation of cancer cells was expressed by the percentage of cell growth inhibition, using the following formula: Inhibitory rate = [ (OD control group - OD experiment group)/OD control group] × 100%.

Clonogenic survival assay

Cells were placed in 6-well plates at a density of 200 cells/well 24 h before the addition of drug or vehicle. Following 48 h of incubation with drug or vehicle, the medium was removed and adherent cells washed with phosphate-buffered saline (PBS) before the culture was continued in a drug-free medium for up to 2 weeks post treatment. Then the medium was removed and the cells were fixed in carbinol and stained with 0.2% crystal violet in PBS. Colonies with >20 cells were visually observed.

Trypan blue assay

Cells treated with penfluridol or vehicle for 48 h were made into a single-cell suspension by trypsin. The suspension was mixed with trypan blue according to the trypan blue staining kit protocol (Beyotime, Shanghai, China). When observed by microscope, the dead cells appeared blue, and live cells appeared colorless. We counted the number of viable cells and dead cells in 3 minutes, and the number of counted cells>300 were divided by time taken. The statistical living cell rate (%) = the number of viable cells/(the number of viable cells and dead cells) × 100%. The data represented the average viability from three separate experiments performed in triplicate. Error bars indicated standard deviations.

Animal tumor models and treatment

We used 5- to 7-week-old female C57BL/6 mice and Babc mice from the Laboratory Animal Center of Sichuan University (Chengdu, China). All studies involving mice were approved by the Institutional Animal Care and Use Committee. C57BL/6 mice were injected subcutaneously in the right axillary fossa with B16 or LL/2 cells (1x10⁶/0.1 ml), while Babc mice were injected subcutaneously in the right axillary fossa with 4T1 or CT26 cells (1x10⁶/0.1 ml). Five to seven days after inoculation, tumors were palpable in the C57BL/6 mice and the Babc mice. Mice were randomly assigned into five groups (n=5 groups for C57BL/6 mice and n=5 groups for Babc mice). Treatments were given via intragastric administration of normal saline (NS), penfluridol (0.06 mg/week), penfluridol (0.12 mg/week), compound cyclophosphamide (0.46 mg/day), and penfluridol (0.06 mg/week) with compound cyclophosphamide (0.46 mg/ day) for 14 days (penfluridol was given on day 1 and on day 8 in the 14 days). The treatment dosage of drugs in the mice was in accordance with the human mouse equivalent dosage conversion (FDA Guidance for industry and reviewers, 2002; FDA Guidance for Industry food-effect bioavailability and fed bioequivalence studies, 2002). Tumor volumes were estimated by the formula length × width²/2.
width"\times 0.52", in which length and perpendicular width were measured by caliper. Subsequently, tissues were subjected to histologic analysis. In a separate survival experiment with three groups each of C57BL/6 mice and Balb c mice (n=7 mice/group), the survival time of the mice was observed to evaluate the life-prolonging effect.

Filipin staining assay

Cancer cells were grown in 24-well plates containing sterile 13 mm glass coverslips at a density of 3x10^6 cells per well. Cells were treated by vehicle or penfluridol at the semi-inhibitory concentration (IC50) for 24 h. After treatment, the cells were washed once with PBS and then stained with filipin according to the protocol of the filipin staining kit (GenMed, Shanghai, China). Intracellular unesterified cholesterol was stained by filipin, which presented blue fluorescence. Coverslips were mounted on slides using ProLong Gold Antifade Reagent (Beyotime, Shanghai, China) and visualized using the Olympus DP70 Digital Camera System (Olympus, Center Valley, PA).

Cholesterol oxidase: p-aminophenazone method (CHOD-PAP)

After treatment for 14 days, we removed the peripheral blood and tumors from the mice. The total serum cholesterol was measured by an auto chemistry analyzer (Hitachi 7100, Tokyo, Japan) according to the protocol of the serum total cholesterol (CHOD-PAP) test kit and the results expressed as mmol/L. The exposed tumors were frozen immediately, and then fully lysed. The tissue total cholesterol from the suspension of the lysate was determined by a protein assay according to the protocol of the tissue total cholesterol (CHOD-PAP) test kit (Polygon Co. Ltd, Beijing, China) by an auto chemistry analyzer (Hitachi 7100, Tokyo, Japan). Also, the protein concentration of the tumors from the mice was determined by a protein assay an auto chemistry analyzer (Hitachi 7100, Tokyo, Japan).

Results

Inhibition of cell proliferation by penfluridol

Penfluridol (C28H27ClF5NO) belongs to the diphenylbutylpiperidine compound; the structure is shown in Figure 1A. The four types of murine cancer cell lines mentioned above were used to evaluate the inhibitory effect of penfluridol on cancer cells. We found that the cellular destiny of each cancer cell line was significantly decreased compared with the control after treatment with 9 \mu mol/L penfluridol for 48 h (Figure 1B). Additionally, penfluridol showed a dose-dependent inhibitory effect on the growth of these cancer cells, as shown in Figure 1C. After B16/F10, LL/2, 4T1 and CT26 cells were treated for 48 h; the IC50 values for penfluridol were approximately 2.51 \mu mol/L, 2.45 \mu mol/L, 3.19 \mu mol/L, 2.74 \mu mol/L respectively. The drug also showed a time-dependent effect. For example, after treatment with penfluridol for 24 h, 48 h and 72 h, the growth of B16/F10 cells was inhibited in a time-dependent manner (Figure 1D).

Cytotoxicity to cancer cells induced by penfluridol in vitro

We performed clonogenic survival assays on B16/F10, LL/2, 4T1 and CT26 cells. These cells were treated with penfluridol for 48 h; this was followed by continuous assessment of cell survival for 2 weeks in drug-free media (Figure 2A). At drug level of 9 \mu mol/L penfluridol, all the cells were dead (no clones observed) 2 weeks after removal of the drug, demonstrating a cytocidal effect of penfluridol in vitro.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using the Social Science (SPSS) version 16.0 (Chicago, Illinois, USA). For comparison of a condition at individual time points, differences between the groups were tested by performing analysis of variance (ANOVA) and an unpaired Student’s test. Survival curves were constructed by the Kaplan-Meier method and statistical significance was determined by the log-rank test. Differences were considered significant at P<0.05.
penfluridol in vitro. This effect was also supported by the result of trypan blue assay. As shown in Figure 2B, more than 90% of the cells in the four types of cancer cell lines were killed by 9 μmol/L penfluridol after 48 h.

Anti-tumor efficacy of penfluridol in vivo

The established B16/F10, LL/2, 4T1, and CT26 cancer models were used to observe the effect of penfluridol on tumor growth in vivo. As shown in Figure 3, compared with the control group, the penfluridol-treated group demonstrated significant inhibition of tumor growth in the LL/2 cancer model. For this cancer on day 21, the tumor volume and tumor weight in the control group were 1514±177 mm$^3$ and 1.08±0.23 g, while in the 0.12 mg/week penfluridol-treated group the values were 608±58 mm$^3$ and 0.52±0.13 g ($p<0.05$). For the B16/F10 and 4T1 cancer models, penfluridol showed a dose-dependent inhibitory effect, with no statistical significance. For CT26, there was less inhibitory effect observed in penfluridol-treated mice. In addition, three other groups of mice (n=7 mice/group) were used to examine the life-prolonging effect of penfluridol (Figure 4). With longer penfluridol treatments, it can be seen that the LL/2 cell lines conferred a greater benefit to mice than other kinds of
relative potency as calmodulin antagonists, and that evidence exists that, for a range of APDs (phenothiazines), the endogenous calcium-binding protein, calmodulin.

One hypothesis is that APDs may have a binding and antagonist action on growth are still not fully understood. One hypothesis is that in skin cancer such as the B16/F10 model and the animal models. The reasons for such responses are unknown. Maybe, in vitro, penfluridol directly interacts with cellular membrane components, as other APDs with known antitumor effects were not prominent in our study. We observed that penfluridol promoted chemotherapy only in the LL2 lung cancer models, while this effect was not observed in other kinds of cancer models (Figure 3).

As is well known, animal cellular growth is dependent on cholesterol. It not only composes cellular membranes and determines many biochemical and biophysical properties of membrane-based processes, but also serves as material for the synthesis of bile acid, vitamin D and steroid hormone. Therefore, maintenance of cholesterol homeostasis is one of the fundamental living requirements for all kinds of cells, including cancer cells. Recently, cholesterol metabolism has become an attractive biochemical target for cancer treatment (Freeman and Solomon, 2004; Llveras et al., 2011). Kristiana et al. reported that in vitro a range of APDs could inhibit cholesterol synthesis in Chinese Hamster Ovary-7 cells with concomitant accumulation of sterol intermediates (Kristiana et al., 2010). They suggested that the dysregulation of cholesterol homeostasis may be an alternative mechanism explanation for APD cytotoxicity to cancer cells; as such drugs induced alteration of those genes that play a major role in regulating cholesterol homeostasis (Wiklund et al., 2010). Our results provided further evidence for this suggestion. As shown in Figures 5 and 6, penfluridol caused unesterified cholesterol to abnormally accumulate in cancer cells in vitro, and, more importantly, it decreased cholesterol content in vivo in a dose-dependent manner in B16/F10, LL2 and 4T1 tumor tissues, which benefited from penfluridol treatment, though not in CT26 tissues, in which there was little anti-tumor effect with penfluridol. Although the exact mechanisms by which penfluridol directly affects cholesterol metabolism and leads to the inhibition of cancer growth were previously unknown, we speculated that dysregulation of cholesterol homeostasis may be involved in its anti-tumor mechanism. In our study, we also found that, despite the fact that penfluridol could induce cholesterol homeostasis disturbance both in vitro and in vivo, there was a difference in effectiveness between in vitro and in vivo treatments, in that penfluridol could cause all cancer cells indicated death in vitro but could not inhibit cancer growth in all the animal models. The reasons for such responses are unknown. Maybe, in vitro, penfluridol directly interacts with cellular membrane components, as other APDs with known anti-tumor effects were not prominent in our study. We observed that penfluridol promoted chemotherapy only in the LL2 lung cancer models, while this effect was not observed in other kinds of cancer models (Figure 3).
In conclusion, the present study demonstrates that penfluridol is not only cytotoxic to cancer cells in vitro but can also inhibit cancer growth in vivo. Dysregulation of cholesterol homeostasis by penfluridol may be involved in this anti-tumor mechanism. This agent may be useful for developing therapeutic regimens for the treatment of cancer.

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References


