RESEARCH ARTICLE

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

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Introduction

Many clinical investigations have found that patients with schizophrenia have a lower risk of cancer than those without (Mortensen, 1989; Dalton et al, 2006). Although the causes of this lower risk remain unclear, certain factors have been considered, such as patients' lifestyle, genetic factors, and whether they receive any antipsychotic drugs (APDs) (Belkin and Hardy, 1957; Mortensen, 1987; Mortensen, 1992; Catts and Catts, 2000; Gal et al., 2012).

As early as 1957, one study reported that psychiatric patients who took APDs had a reduced risk of cancer. Further research found that certain APDs, such as phenothiazines, can inhibit the growth of some human cancer cells (Wuonola et al., 1998; Zhelev et al., 2004; Gil-Ad et al., 2004). Recently, Yeh et al. found that trifluoperazine can inhibit the growth of lung cancer stem cells (Yeh et al., 2012). Sachlos et al. also found that thioridazine can inhibit human leukemia stem cell transformation (Sachlos et al., 2012). These data suggest that APDs might have a potential anti-tumor value for clinical treatment.

However, the mechanisms whereby APDs may inhibit tumor cell growth are not yet fully understood. There may be a variety of explanations (Nordenberg et al., 1999). For example, some studies have shown that anti-sigma receptors might provide a mechanism for APD toxicity to tumor cells (Rybczynska et al., 2008); others have shown that the mechanism of against to calmodulin might contribute to the cytotoxicity of APDs, such as phenothiazine and trifluoperazine (Levin and Weiss, 1977; Hait and Lee, 1985). Another alternative explanation is that the APDs could modify plasma membrane properties related to their cationic amphiphilic properties, which may lead to a dysregulation of cell cholesterol homeostasis (Lange and Steck, 1994; Cheung et al., 2004; Murata et al., 2007; Suwalsky et al., 2008). Recently, Erik found that cholesterol synthesis genes and mRNA appeared to

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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); serum total cholesterol (CHOD-PAP) test kit (Maker Co. Ltd, Chengdu, China); tissue total cholesterol (CHOD-PAP) test kit (Polygon Co. Ltd, Beijing, 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 fatal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin in a humid chamber at 37°C, 5% CO_2 in atmosphere.

Cell viability assay

The cell viability of penfluridol-treated cancer cells was determined by the MTT assay. Briefly, cells (4-5×10^3) 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 viability 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 Bablc 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 (1×10^6/0.1 ml), while Bablc mice were injected subcutaneously in the right axillary fossa with 4T1 or CT26 cells (1×10^6/0.1 ml). Five to seven days after inoculation, tumors were palpable in the C57BL/6 mice and the Bablc mice. Mice were randomly assigned into five groups (n=5 groups for C57BL/6 mice and n=5 groups for Bablc 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 ×

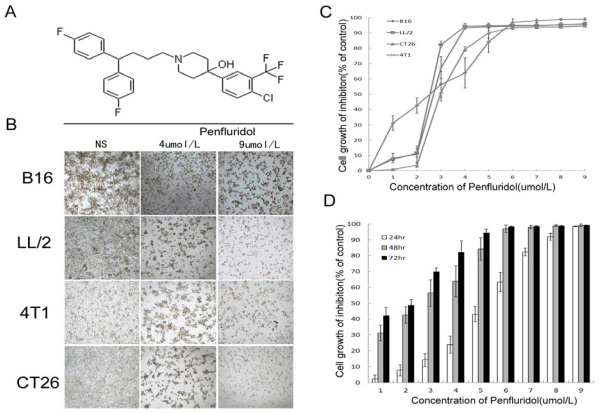

Figure 1. Penfluridol Inhibited Cancer Cells Growth in Vitro. (A) Structure of penfiuidol. (B) Representative cell density changes of B16/F10, LL/2, 4T1 and CT26 cells treated with penfiuidol at different concentrations for 48 h. (C) penfiuidol -inhibited proliferation of cancer cells in a dose-dependent manner. (D) penfiuidol- inhibited proliferation of B16 cancer cells in a time-dependent manner. Results of MTT assays are expressed as mean ± SD of 6 wells in triplicate experiments

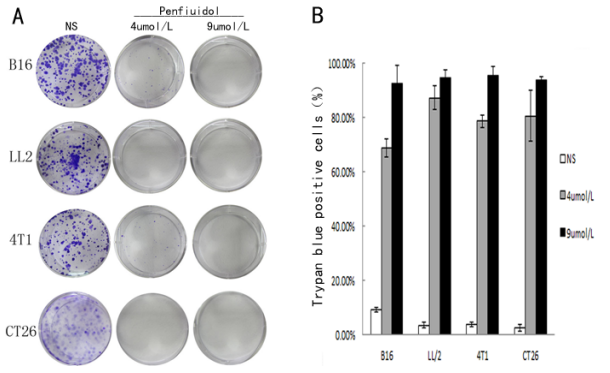

Figure 2. Cytotoxicity of Penfluridol to Cancer Cells. (A) Very few cells survived in B16, LL/2, 4T1 and CT26 cells after treated with 4 μmol/L and 9 μmol/L penfluridol for 48h. (B) Trypan blue assays showed the number of dead cells significantly increased as compared with the control after treated with penfluridol for 48h. Results of trypan blue assays are expressed as mean ± SD from 3 separate experiments performed in triplicate experiments

width^2 × 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 Bablc 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 3×10^4 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 measured 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 suspension of the lysate was determined by a protein assay kit (Beyotime, Shanghai, China). The tissue cholesterol measurements were expressed as mmol/g, which equated to the relative cholesterol content of the protein.

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 $P<0.05$.

Results

Inhibition of cell proliferation by penfluridol

Penfluridol ($C_{28}H_{27}ClF_5NO$) 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 μmol/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 μmol/L, 2.45 μmol/L, 3.19 μmol/L, 2.74 μmol/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 μmol/L penfluridol, all the cells were dead (no clones observed) 2 weeks after removal of the drug, demonstrating a cytocidal effect of

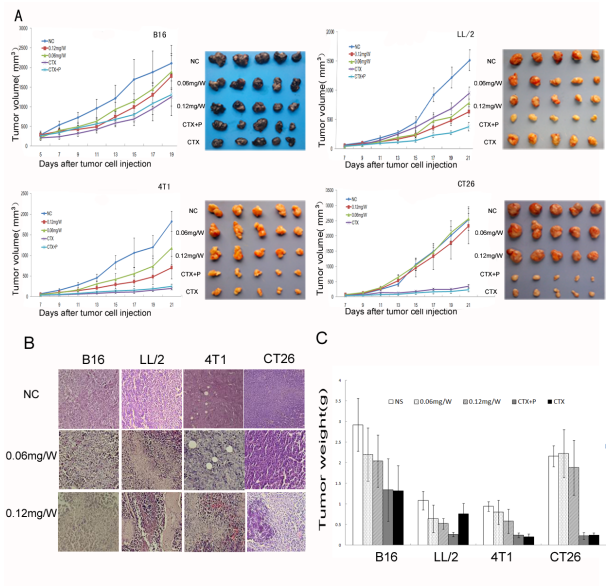

Figure 3. Inhibition of Tumor Growth by Penfluridol in Vivo. Mice were treated intragastric administration of NS, penfluridol (0.06 mg/week), penfluridol (0.12 mg/week), compound cyclophosphamide (0.46 mg/day), and penfluridol (0.06 mg/week) with compound cyclophosphamide (0.46 mg/day) for 14 days. (A) Representative antitumor efficacy of penfluridol presented by tumor volume in the four tumor models (n=5/group). The results were presented by the mean volume ± SD. (B) Micrographs of hematoxylin/eosin-stained sections of tumor tissues from penfluridol-treated mice (0.06 mg/week), penfluridol-treated mice (0.12 mg/week) and NS mice. There were more necrosis areas in tumor in B16/F10, LL/2 and 4T1 penfluridol -treated group as compared with that in nature control (NC) group. (C) Antitumor efficacy of penfluridol presented by tumor weight in the four tumor models (n=5). The results were showed by the mean weight ± SD

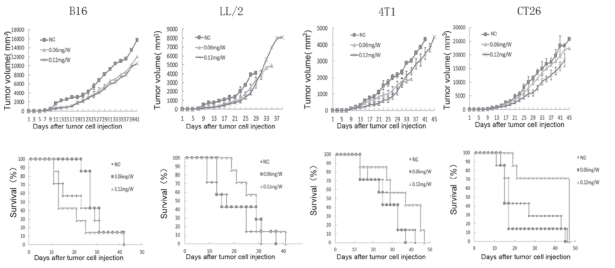

Figure 4. Life-prolonging Effect Mediated by Penfluridol. Results showed that although tumor volume of mice (n=7/group) in penfluridol -treated groups was smaller than that in the NS-treated ones, the significant life-prolonging effect was only observed in LL/2 cancer model. Tumor volume was presented as mean ± standard. When the remaining number of mice ≤ 2 in one group, results of it were just expressed as the mean

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

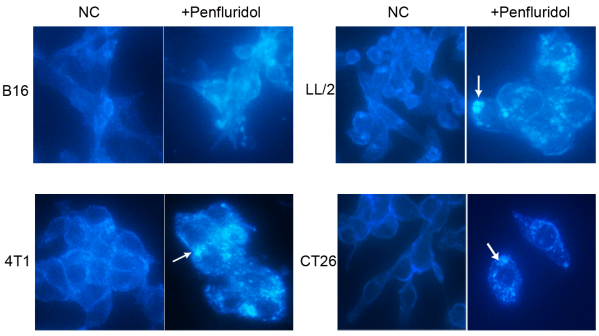

Figure 5. Unesterified Cholesterol Accumulated in Cancer Cells *in Vitro*. Cancer cells indicated were given at IC50 concentrations of penfluridol respectively for 24h, and then stained by filipin. Unesterified cholesterol presented highlight blue fluorescence (white arrows point to, for instance) was observed via fluorescence microscopy. The experiment was repeated in three times, each time>100 cells observed .It appeared accumulation of unesterified cholesterol in cancer cells after treated with penfluridol, and this phenomenon is much more obvious in LL/2, 4T1, and CT26 cells

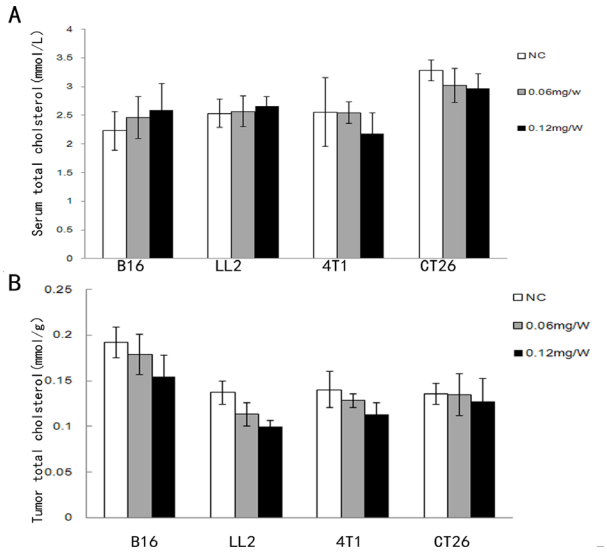

Figure 6. Penfluridol Affected Cholesterol Metabolism in Vivo. Mice were taken from NS group, penfluridol (0.06 mg/week) group, and penfluridol (0.12 mg/week) group, and then their serum total cholesterol and tumor total cholesterol were detected using CHOD-PAP assay. (A) In serum total cholesterol content, there was no significant difference between the NS-treated mice and penfluridol-treated mice. (B) In tumor tissues, total cholesterol content showed a dose-dependent decrease in B16/F10, LL/2, and 4T1 models treated with penfluridol

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

cancer models. From Figure 4, it can also be seen that all NS-treated mice of the LL/2 model died of tumor burden within 30 days after implantation, and that 0.12 mg/week penfluridol-treated mice showed a significantly prolonged survival time.

Dysregulation of cholesterol homeostasis by penfluridol in vitro and in vivo

Recently some studies have reported that a range of APDs can dysregulate cholesterol homeostasis in vitro and that this would be a novel chemotherapeutic target (Wiklund, 2010). In this study, we found that unesterified cholesterol had accumulated in cells after treatment with penfluridol for 24 h in vitro (Figure 5). In vivo, no significant difference in serum cholesterol levels was found between the NS group and the penfluridol-treated group (Figure 6A). However, a dose-dependent decrease of total cholesterol in tumor tissues was observed in the penfluridol-treated group (Figure 6B). Phenomenon marked effect of decreased total cholesterol was observed in the B16/F10, LL/2 and 4T1 tumor models, which also benefited from penfluridol treatment (Figures 1 and 2).

Discussion

Psychiatric patients have a lower risk of cancer than non-psychiatric patients (Mortensen, 1989; Dalton et al., 2006). Certain APDs have an effect on inhibiting tumor growth (Mortensen, 1992; Gil-Ad et al., 2004; Yeh et al., 2012). Penfluridol, a stronger APD, might also have such an anti-tumor effect.

In the present study, we found that penfluridol can inhibit tumor growth in vitro and in vivo. Penfluridol alone can inhibit the growth of a variety of murine solid cancer cells in vitro in both a dose-dependent and time-dependent manner (Figure 1). The cytotoxic potential of penfluridol to cancer cells was demonstrated by clonogenic assays and trypan blue staining assays (Figure 2). Moreover, tumor sensitivity to penfluridol varies between cancers (B16/F10, LL/2, 4T1, CT26) in vivo (Figure 3). For lung cancer cells, penfluridol shows a stonger inhibitory effect on tumor growth, and the survival time of tumor-bearing mice is extended (Figure 4). Meanwhile, penfluridol has little anti-tumor effect on colon cancer CT26 in vivo (The difference was not statistically significant). Compared with the control group, the tumor volume and tumor weight in the penfluridol-treated group were lower than that in skin cancer such as the B16/F10 model and the breast cancer 4T1 model, although there was no statistical significance. This anti-tumor efficacy of penfluridol in vivo varied according to the tissue origin of the cancer. These findings suggest that the targeting of certain types of cancer (such as lung cancer) with penfluridol may be a useful approach for cancer therapy.

The mechanisms by which APDs inhibit tumor cell growth are still not fully understood. One hypothesis is that APDs may have a binding and antagonist action on the endogenous calcium-binding protein, calmodulin. Evidence exists that, for a range of APDs (phenothiazines), the strength of their cytotoxic effect corresponds to their relative potency as calmodulin antagonists, and that calmodulin antagonists have an effect on reversing multi-drug resistance and have a similar synergistic effect with anti-cancer chemotherapeutic agents (Levin and Weiss, 1977; Hait and Lee, 1985). However, these synergistic effects were not prominent in our study. We observed that penfluridol promoted chemotherapy only in the LL/2 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; Llaverias 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, LL/2 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 , which leads to further cell death as the properties of the cellular membranes are abnormally altered (Murata et al., 2007; Maruoka et al., 2007; Steinkopf et al., 2008; Suwalsky et al., 2008). This capacity might be partly explained by the fact that the drugs disrupt cholesterol delivery, which accommodates cholesterol homeostasis. In vivo, penfluridol selectively inhibits cholesterol synthesis in some cancers, and this selectivity probably depends on the sensitivity of the cancer to different drugs, as, for example, research suggests that lung cancer is more sensitive to APD treatment (Solomon and Freeman, 2008; Yeh et al., 2012).

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 regimen for the treatment of cancer.

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