

Original Research

Antipsychotic Zuclopenthixol Inhibits Melanoma Growth and Brain Metastasis by Inducing Apoptosis and Cell Cycle Arrest

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Abstract

Background: The incidence of melanoma brain metastasis (MBM) is high and significantly compromises patient survival and quality of life. Effective treatment of MBM is made difficult by the blood-brain barrier (BBB), since it restricts the entry of drugs into the brain. Certain anti-psychotic drugs able to cross the BBB have demonstrated efficacy in suppressing brain metastasis in preclinical studies. However, the activity of zuclopenthixol against MBM is not yet clear. **Methods**: Cell viability assays were employed to investigate the potential of zuclopenthixol in the treatment of MBM. Subsequently, the mechanism of action was investigated by RNA-sequencing (RNAseq), flow cytometry-based cell cycle and apoptosis assays, protein expression analysis, and autophagy flux detection. Additionally, the efficacy of zuclopenthixol against tumor growth was investigated *in vivo*, including MBM models. **Results**: Zuclopenthixol inhibited the proliferation of various melanoma cell lines at minimal doses by causing cell cycle arrest in the G0/G1 phase and mitochondrial-mediated intrinsic apoptosis. Zuclopenthixol also induced cytoprotective autophagy, and inhibition of autophagy enhanced the anti-melanoma effects of zuclopenthixol. Furthermore, zuclopenthixol inhibited the growth of human melanoma tumors in nude mice, as well as the growth of intracranial metastases in a mouse model of MBM. **Conclusions**: These results demonstrate that zuclopenthixol has significant potential as an effective therapeutic agent for MBM.

Keywords: zuclopenthixol; cell cycle arrest; brain metastasis; apoptosis; melanoma

1. Introduction

Since most patients with solid cancer succumb to metastasis, inhibition of this process is crucial for improving cancer patient survival. Late-stage melanoma exhibits strong metastatic behavior, with the brain, liver, lung, bone, and peritoneum being the primary sites of metastasis [1]. Limited information on the treatment progress of melanoma brain metastases (MBM) is available, mainly due to the exclusion of patients with brain metastases from clinical trials. However, recent advances in targeted therapies and immunotherapies have significantly improved the treatment of extracranial melanoma [2]. Positive outcomes have also been observed for the treatment of MBM, with combined inhibition of serine/threonine-protein kinase B-Raf(BRAF) and mitogen-activated protein kinase kinase (MEK) leading to intracranial response rates of almost 60% [3]. The combination of nivolumab and ipilimumab also demonstrated a clinical benefit in 58.4% of patients with symptomatic MBM [4]. Despite these advances, some MBM patients do not respond favorably to treatment, while others experience relapse after an initial response. Overall, the survival of MBM patients has not been significantly prolonged, with a median overall survival ranging from 5.6 to 17.4 months [5]. Consequently, melanoma metastases, particularly fatal MBM, continue to present significant challenges for the effective treatment of advanced melanoma [6].

A major challenge in the treatment of brain metastases is the blood-brain barrier (BBB), which hinders drug entry and promotes brain efflux of anti-cancer drugs [7]. Moreover, the development of new anti-cancer drugs is limited by high costs, lengthy timelines, and elevated risks of failure [8]. Consequently, the repurposing of existing drugs has attracted the attention of academic researchers and pharmaceutical companies in recent years. Comprehensive data is already available for approved drugs, including clear targets, mechanism of action, and safety profiles [9]. Once approved drugs are identified as being suitable for standalone treatments or for synergistic effects with other approved drugs, they can be efficiently repurposed for other diseases. Successful examples of this in the cancer field include the antiemetic drug thalidomide for the treatment of multiple myeloma, and the immunosuppressive drug rapamycin used in the treatment of colorectal cancer [10].

Previous studies have suggested that patients using anti-psychotic drugs have a lower cancer incidence, suggesting these drugs may also have anti-cancer properties [11,12]. Recent studies, including our previous research, showed that certain anti-psychotic medications effectively



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impede the growth and spread of cancer cells, both *in vitro* and *in vivo*. For example, penfluridol has been shown to inhibit various cancer types, including lung [13], esophageal [14], breast [15] and renal [16], while trifluoperazine has been shown to inhibit triple-negative breast cancer [17] and colorectal cancer [18]. The ability of anti-psychotic drugs to cross the BBB makes them promising candidates for treating brain metastasis. Therefore, the investigation of potential anti-cancer drugs amongst anti-psychotic medications, especially those that inhibit brain metastasis, may be quite productive.

Zuclopenthixol is a typical anti-psychotic and acts as a D1 and D2 dopamine receptor antagonist, as well as an α 1-adrenergic and 5-hydroxytryptamine 2 (5-HT2) receptor antagonist. Some studies have reported on the potential of zuclopenthixol for the treatment of breast and esophageal cancers [19,20]. B16 melanoma is a malignant murine tumor cell line widely used as a model for human skin cancer. In a previous study, we examined the inhibitory effect of several anti-psychotic drugs on B16 cell growth. Zuclopenthixol was found to show promising activity against this aggressive cell line [21]. However, its effects on MBM and the underlying mechanism are still not known. The aim of this study was therefore to investigate the possibility of repurposing zuclopenthixol for the treatment of melanoma and MBM, thus offering a potential new therapeutic option. This research sought to examine the effects and underlying mechanisms of zuclopenthixol on the proliferation of melanoma and on its spread to the brain.

2. Materials and Methods

2.1 Materials

Zuclopenthixol (Cat# G12762) was purchased from AstaTech BioPharmaceutical Corporation (Chengdu, China), the Annexin V-7-AAD dual-staining apoptosis detection kit (Cat# 420404) from Biolegand (San Diego, CA, USA), propidium iodide (PI, Cat# ST1569-10mg) and DCFH-DA (Cat# S0033S) from Beyotime (Shanghai, China), and Rhodamine 123 (Cat# R8004-5MG) and MTT (Cat# M5655-1G) from Sigma-Aldrich (St Louis, MO, USA). 3-methyladenine (3-MA, Cat# M129496-1g) and chloroquine diphosphate (CQ, Cat# C129284-5g) from Aladdin (Shanghai, China).

2.2 Cell Lines and Cell Culture

The melanoma cell lines B16, A375, SK-MEL-28, C32, W266-4, A875, HT144, SK-MEL-2, WM115, and A2058 were bought from the American Type Culture Collection (ATCC) within 5 years. These cell lines were grown in DMEM culture medium containing 100 U/mL of penicillin and streptomycin, and 10% FBS. The cells were maintained at 37 °C under humidified conditions and with 5% CO_2 . All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.3 Cell Viability and Colony Formation Assays

The impact of zuclopenthixol on melanoma cell viability was assessed using the MTT assay. Between 1000 to 3000 cells were placed in a 96-well plate for 24 h, after which an increasing amount of zuclopenthixol was added to individual wells and incubated for different times. Subsequently, 20 μ L of MTT solution (5 mg/mL) was added, and the cells incubated at 37 °C for 2–3 h. Next, the medium was removed and 150 μ L of DMSO was added to each well. The absorbance at 562 nm was then measured, thus allowing the rate of inhibition to be calculated for the various concentrations of zuclopenthixol. Half-maximal inhibitory concentration (IC₅₀) values were determined using Graph-Pad Prism 5 software (version 8.0.1, GraphPad Software, Boston, MA, USA).

The colony formation assay was conducted using 6well plates. Each well was seeded with 800 cells and these were subjected to varying concentrations of zuclopenthixol for a period of 7 to 10 days. The treatment duration for B16 cells was 7 days, while for A375 cells it was 10 days. Cells were then fixed with a 0.5% solution of crystal violet, and the number of colonies counted under a microscope to determine the rate of inhibition.

2.4 Transcriptome Sequencing (RNA-Seq)

Following treatment with vehicle (0.1% DMSO) or zuclopenthixol (12.5 µM) for 24 h, A375 cells were processed using TRIzol (Invitrogen, Carlsbad, CA, USA) in order to obtain total RNA. Three samples were harvested for each treatment group. RNA purity and concentration were evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), while RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). PolyA selection with oligo(dT) beads was performed to isolate mRNA. The SuperScript kit (Invitrogen) was used to synthesize cDNA using random hexamer primers purchased from Illumina (San Diego, CA, USA). Sequencing and analysis of the transcriptome was carried out by Majorbio Co. Ltd. (Shanghai, China). The libraries were sequenced on the Illumina Novaseq 6000 platform. Genes exhibiting a $|\log_2 FC| \ge 1$ and an adjusted *p*-value < 0.05 were classified as differentially expressed genes (DEGs). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs and Reactome annotations were performed using R (version 3.2.0) by Majorbio Co., Ltd. (Shanghai, China).

2.5 Analysis of Cell Cycle Distribution and Apoptosis

For analysis of the cell cycle, melanoma cells were treated with zuclopenthixol for 12 h and 24 h, followed by fixation for 24 h in ice-cold 75% ethanol. Cells were then rinsed with PBS and placed in the dark for 30 minutes in a solution containing RNase, 50 μ g/mL of propidium iodide (PI), and 0.1% Triton X-100. After washing in PBS, the cell cycle distribution was examined using ACEA Novo-

Cyte and analyzed using NovoExpress software (version 1.4.1.1901, ACEA Biosciences Inc., San Diego, CA, USA).

The detection kit for analysis of apoptosis used Annexin V-7-AAD dual-staining. The cells were placed in 6-well dishes at a concentration of 1×10^5 cells per well and exposed to zuclopenthixol for 24 h. Following staining with Annexin V-PE and 7-AAD, flow cytometry (FCM) was performed to measure the levels of apoptosis. NovoExpress software was used to analyze apoptosis in different treatment groups.

2.6 Assessment of the Potential Difference across the Mitochondrial Membrane ($\Delta \Psi m$) and the Presence of Reactive Oxygen Species (ROS)

Increasing amounts of zuclopenthixol were administered to melanoma cells for 24 h, followed by assessment of $\Delta\Psi$ m and ROS. The cells were exposed to 5 µg/mL of Rh123 at 37 °C in the dark for 30 minutes to measure $\Delta\Psi$ m. After washing with PBS, cells were analyzed by FCM to evaluate the decrease in $\Delta\Psi$ m based on the fluorescence intensity of the fluorescein isothiocyanate (FITC) channel. To measure ROS, cells were treated with 10 µM Dichlorodihydro-fluorescein diacetate (DCFH-DA) and incubated for 30 minutes at 37 °C in the dark. After washing with PBS, cells were examined by FCM to determine the ROS content based on the fluorescence intensity of the FITC channel.

2.7 Transwell Migration and Cell Invasion

For the migration test, 5×10^4 B16 cells or 1×10^4 A375 cells were placed in the upper compartment of a transwell with 200 µL of DMEM medium without serum. The lower compartment was filled with 600 µL of medium containing 10% FBS and zuclopenthixol. For the invasion assay, 60 µL of diluted matrigel (matrigel:PBS = 1:5) was added to the top chamber of the transwell. Following incubation at 37 °C for 1 h, the upper compartment received serum-free medium containing 2×10^5 B16 cells or 2.5×10^5 A375 cells, while the lower compartment received 600 µL of medium containing 10% FBS and zuclopenthixol. After 24 h, cells that moved through the transwell were immobilized with 4% paraformaldehyde and stained with 0.5% crystal violet for quantification using a microscope.

2.8 Western Blot Analysis

After treatment with different concentrations of zuclopenthixol for 24 h, proteins were extracted by lysing cells with a lysis buffer containing a protease inhibitor cocktail. Proteins were then separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto nitrocellulose membranes (Millipore). Subsequently, conventional Western blot analysis was performed to detect protein expression. The antibodies used for Western blot analysis were as follows: anti-DRD-2 was

purchased from Abclonal (Wuhan, China). Anti-p62 (Cat#CY5546, WB: 1:6000 dilution), anti-p-p44/42 MAPK (Thr202/Tyr204) (Cat#CY5044, WB: 1:2000 dilution), anti-CDK2 (Cat#CY5020, WB: 1:1000 dilution), and anti-GAPDH (HRP conjugated) (Cat#AB2000, WB: 1:20,000 dilution) were purchased from Abways (Shanghai, China). Anti-Bax (Cat#610982, WB: 1:1000 dilution) and anti-Bcl2 (Cat#2610538, WB: 1:1000 dilution) were purchased from BD Bioscience (Piscataway, NJ, USA). Anti-AKT (Cat#4685s, WB: 1:1000 dilution), anti-p-AKT (Cat#4060s, WB: 1:2000 dilution), anti-cyclin D1 (Cat#2978, WB: 1:1000 dilution), anti-p27 (Cat#3698, WB: 1:1000 dilution), anti-cleaved caspase 3 (Cat#9664s, WB: 1:1000 dilution), anti-p44/42 MAPK (Cat#4696, WB: 1:2000 dilution), and anti-p-yH2AX (Cat# 9718S, WB: 1:1000 dilution) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-LC3 (Cat#NB100-2220, WB: 1:1000 dilution) was sourced from NOVUS (Littleton, CO, USA). Anti-β-actin (Cat#200068-8F10, WB: 1:10000 dilution), anti-LC3 (Cat#NB100-2220, WB: 1:1000 dilution), and anti-CDK4 (Cat# 200540, WB: 1:1000 dilution) were purchased from Zen Bioscience (Chengdu, China). The intensity of bands was quantified using ImageJ software (NIH, Bethesda, MD, USA).

2.9 Plasmid Transfection and Immunofluorescent Staining

Transfection of the GFP-RFP-LC3 plasmid into A375 cells was performed using Lipofectamine 3000 (Invitrogen). Cells were placed on Millicell EZ slides (Merck Millipore, Darmstadt, Germany) for 24 h and then exposed to zuclopenthixol (12.5 μ M) or CQ (20 μ M) for 24 h. They were subsequently immobilized with 4% paraformaldehyde at room temperature for 30 minutes and stained with 4',6diamidino-2-phenylindole (DAPI) solution (5 μ g/mL) for 5 minutes. Confocal microscopy (LSM 880, Zeiss Microsystems, Jena, Germany) was utilized to observe the presence of LC3 in cells.

2.10 Establishment of Subcutaneous Melanoma and Experimental Brain Metastasis Models

Approval for the ethical use of animal models was obtained from the Animal Care and Use Committee, Sichuan University. In order to create the xenograft model for subcutaneous melanoma, 5×10^6 A375 cells were injected into the right flank of female BALB/c nude mice aged 8– 10 weeks and purchased from HFK Bioscience (Beijing, China). Upon reaching an average tumor volume of approximately 100 mm³, the mice were randomly divided into either zuclopenthixol (40 mg/kg) or control groups. The vehicle solution was a mixture of 5% DMSO, 12.5% Cremophor EL, and 82.5% regular saline. Treatments were administered via intraperitoneal injection and the tumor dimension (length and width) and body weight were recorded every 3 days. For the *in vivo* model of MBM, 1×10^4 B16 cells expressing luciferase were injected into the carotid, as previously described [12]. After four days of inoculation, treatment was begun with either zuclopenthixol (40 mg/kg) or vehicle, which were administered by intraperitoneal injection once every day. Brain tumor signals were quantified utilizing a non-invasive *in vivo* imaging system (IVIS, Perkin Elmer, Waltham, MA, USA).

2.11 Statistical Analysis

Data is shown as the average \pm standard deviation, and statistical analysis was performed using the Graph-Pad Prism 5 software (version 8.0.1, GraphPad Software, Boston, MA, USA). The two-tailed Student's *t*-test was performed to compare two groups and assess statistical significance. One-way Analysis of Variance (ANOVA) was utilized for comparison of multiple groups, followed by Dunnett's test.

3. Results

3.1. Inhibitory Effect of Zuclopenthixol on Melanoma Cells in Vitro

In our previous study [21], zuclopenthixol showed strong inhibitory activity against B16 cells, prompting us to expand testing to 9 other melanoma cell lines. Interestingly, zuclopenthixol inhibited all melanoma cell lines examined, with IC_{50} values ${<}15\,\mu\text{M}$ after a 48 h treatment period (Fig. 1A). We next investigated the activity and mechanism of zuclopenthixol against human (A375) and mouse (B16) melanoma cell lines. As shown in Fig. 1B,C, zuclopenthixol suppressed the viability of B16 and A375 cells in a time- and concentration-dependent manner. Colony formation experiments revealed that low concentrations of zuclopenthixol reduced the number of melanoma cells (Fig. 1D,E). We also assessed the impact of zuclopenthixol on the phosphorylation of p44/42 MAPK and AKT. The results indicated effective inhibition of MAPK and AKT activation by zuclopenthixol in both cell lines (Fig. 1F). Zuclopenthixol is a dopamine receptor D2 (DRD2) antagonist. Interestingly, in vitro treatment with 10 µM and 15 µM zuclopenthixol reduced DRD2 expression in A375 cells, but not in B16 cells (Fig. 1G). Taken together, these results suggest that zuclopenthixol can significantly inhibit the viability of melanoma cells at low concentrations in vitro, thus warranting further evaluation of its inhibitory effects on melanoma.

3.2 Zuclopenthixol Induces G0/G1 Phase Arrest in Melanoma Cells

We conducted RNA-Seq analysis to study the molecular mechanism by which zuclopenthixol inhibits melanoma. Following treatment of A375 cells with zuclopenthixol, the expression of 1047 genes was found to be upregulated, while that of 295 genes was downregulated (Fig. 2A,B). Reactome annotation analysis indicated that one of the enriched pathways was the cell cycle (Fig. 2C).

The ability of zuclopenthixol to inhibit the viability of melanoma cells may be due to its capacity to impede progression of the cell cycle and/or provoke cell demise. We therefore examined the effect of zuclopenthixol on the cell cycle of melanoma cells. In both cell lines, treatment with zuclopenthixol for 12 and 24 h led to a notable increase in the percentage of cells in the G0/G1 stage, potentially decreasing the S and G2/M phases (Fig. 3A-D). We also analyzed the expression of several important regulators of the cell cycle, including cyclin D1, CDK2 and CDK4. These proteins play crucial roles in promoting the G1-S transition, which was suppressed by zuclopenthixol treatment (Fig. 3E,F). Furthermore, zuclopenthixol increased the expression of p27. This inhibits cyclin-CDK and halts progression of the cell cycle in the G1 phase, thus preventing initiation of the normal cell cycle. These findings demonstrate that zuclopenthixol may exert its anti-tumor effects in melanoma cells in part by blocking G0/G1 phase progression.

3.3 Zuclopenthixol Causes Mitochondrial-Dependent Apoptosis in Melanoma Cells

KEGG analysis of the DEGs identified after zuclopenthixol treatment indicated enrichment for pathways related to cell growth and death (Fig. 4A). This implies that the mechanism for zuclopenthixol inhibition of melanoma may involve cell demise. Subsequent FCM analysis revealed that treatment with zuclopenthixol induced significant apoptosis of melanoma cells (Fig. 4B). Western blot analysis showed that administration of zuclopenthixol resulted in upregulation of cleaved caspase 3, an essential factor in apoptosis (Fig. 4C). Additionally, zuclopenthixol markedly increased the expression of phosphorylated γ H2Ax, indicating it can induce DNA fragmentation leading to apoptosis (Fig. 4C). Collectively, these findings suggest that zuclopenthixol-induced apoptosis may contribute to the inhibitory effects of this drug on melanoma.

Two primary categories of apoptosis are currently recognized. The first is the extrinsic apoptosis pathway mediated by cell death receptors, and the second is the intrinsic apoptosis pathway mediated by mitochondria. Various drugs induce apoptosis in tumor cells through the intrinsic pathway of programmed cell death. To investigate whether this pathway is involved in zuclopenthixol-induced melanoma apoptosis, we examined the expression of important regulatory proteins within the BCL2 family, as these play crucial roles in maintaining mitochondrial integrity. Treatment with zuclopenthixol significantly upregulated the expression of Bax, while simultaneously downregulating the expression of BCL2 (Fig. 4C). Zuclopenthixol also decreased the $\Delta \Psi m$ in melanoma cells (Fig. 5A) and increased the intracellular ROS level (Fig. 5B). Moreover, the antioxidant glutathione (GSH) strongly reversed the apoptosis induced by zuclopenthixol (Fig. 5C). Together, these



Fig. 1. Zuclopenthixol suppresses melanoma growth and viability *in vitro.* (A) The Half-maximal inhibitory concentration (IC_{50}) value of zuclopenthixol was evaluated in 10 melanoma cell lines after 48 h of treatment. (B,C) The effect of zuclopenthixol on the growth of A375 and B16 cells was assessed by measuring their viability after treatment with various concentrations of the drug for 24 and 48 h. (D,E) The effect of zuclopenthixol on the ability of A375 and B16 cells to form colonies was evaluated following 7 days of treatment for B16 cells and 10 days for A375 cells. Quantitative results are shown on the right side of representative images. (F) Effect of zuclopenthixol on the phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) in A375 and B16 cells after 24 h of treatment. (G) Effect of zuclopenthixol on the expression level of dopamine receptor D2 (DRD2) in A375 and B16 cells after 24 h of treatment. ***p*-values < 0.01 are considered highly significant compared to the vehicle group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



Fig. 2. The effect of zuclopenthixol on gene expression in A375 cells. (A) The number of genes upregulated and downregulated after 12.5 μ M zuclopenthixol treatment for 24 h. Differentially expressed genes (DEGs) were identified as genes with a log₂ fold change >2 and an adjusted *p*-value < 0.05. (B) Heatmap displaying the DEGs identified through RNA-sequencing (RNA-seq) analysis of A375 cells after 24 h treatment with or without zuclopenthixol. (C) Reactome annotations were used to analyze the DEGs.



Fig. 3. Zuclopenthixol suppresses cell cycle progression in melanoma cells. (A,C) Flow cytometry (FCM) was used to evaluate the distribution of A375 and B16 cells in distinct phases of the cell cycle following exposure to different doses of zuclopenthixol for 12 and 24 h. (B,D) Quantitative analysis of the results shown in A and C. (E,F) The impact of treatment with zuclopenthixol for 24 h on the expression of proteins that regulate the G1/S transition in A375 and B16 cells. **p*-values < 0.05 are considered significant, while ***p*-values < 0.01 are considered highly significant compared to the vehicle group.



Fig. 4. Melanoma cells undergo apoptosis when exposed to zuclopenthixol. (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs. (B) Effect of zuclopenthixol on melanoma cell apoptosis. FCM was used to analyze the apoptosis of A375 and B16 cells after treatment with various concentrations of zuclopenthixol for 24 h. (C) Apoptosis-associated protein expression in cells evaluated by Western blot after 24 h treatment with zuclopenthixol. **p*-value < 0.05, ***p*-value < 0.01, and ****p* value < 0.001 compared to the vehicle group.

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results indicate the intrinsic apoptosis pathway mediated by mitochondria is essential for the induction of apoptosis in melanoma cells by zuclopenthixol.

3.4 Zuclopenthixol Triggers Protective Autophagy and Inhibits the Flow of Autophagy in Melanoma Cells

Analysis of the Reactome annotations in A375 cells revealed enrichment for autophagy following treatment with zuclopenthixol (Fig. 6A). We therefore speculated that zuclopenthixol might regulate autophagy in melanoma cells. Autophagy markers include the conversion of LC3-I to LC3-II, and the development of LC3 puncta. Treatment of melanoma cells with zuclopenthixol increased the conversion of LC3-I to LC3-II (Fig. 6A), as well as increasing the level of p62, a specific substrate for autophagy and an indicator of autophagy flux (Fig. 6A). These results indicate that zuclopenthixol can hinder the flow of autophagy. To determine whether this may be caused by the deficient fusion of autophagosomes with lysosomes, an LC3B construct labeled with tandem red fluorescent protein-green fluorescent protein (RFP-GFP) was utilized. The majority of LC3B puncta showed signal for RFP+GFP+ (autophagosomes) rather than RFP+GFP- (autolysosomes) (Fig. 6B). Therefore, treatment with zuclopenthixol results in the accumulation of autophagosomes, a reduction in the formation of autolysosomes, and the accumulation of LC3 puncta.

The above findings indicate that zuclopenthixol may impede autophagy flux in melanoma cells. To better understand the mechanism by which zuclopenthixol exerts antimelanoma effects, it was administered to A375 and B16 cells together with two frequently employed autophagy inhibitors. These were chloroquine (CQ), which hinders the fusion of autophagosomes with lysosomes at a late stage, and 3-methyladenine (3-MA), which is an early-stage inhibitor that impedes PI3K activity. The growth of A375 and B16 cells was significantly inhibited by the combination of zuclopenthixol and both autophagy inhibitors, as shown in FFig. 6C,D. Similarly, the combination of zuclopenthixol and autophagy inhibitors significantly increased apoptosis in these cells (Fig. 6E,F). Overall, zuclopenthixol triggers a defensive process of self-degradation in cells. Therefore, additional suppression of this process could potentially strengthen the ability of zuclopenthixol to inhibit melanoma cells.

3.5. Effects of Zuclopenthixol on Subcutaneous Melanoma Tumor Growth and Brain Metastasis in Mice Models

After establishing the subcutaneous melanoma model with A375 cells in BALB/c nude mice, we investigated the *in vivo* efficacy of zuclopenthixol for inhibiting melanoma growth. Zuclopenthixol effectively inhibited the growth of melanoma in mice, without causing a notable effect on their body weight (Fig. 7A). These findings highlight the potential of zuclopenthixol as a treatment drug for melanoma. Given its ability to penetrate the BBB, we hypothesized that zuclopenthixol may also hold promise for treating MBM. To explore this, we initially assessed its capacity to inhibit the migration and invasion of B16 and A375 cells, two critical processes involved in cancer metastasis. Zuclopenthixol significantly suppressed both processes *in vitro*, indicating that it could potentially suppress metastasis *in vivo* (Fig. 7B). In a mouse model of B16 cell brain metastasis, zuclopenthixol strongly inhibited intracranial metastatic tumor growth (Fig. 7C,D), leading to prolonged survival of mice (Fig. 7E). Of note, treatment with zuclopenthixol did not significantly decrease mouse body weight (Fig. 7F), thus providing preliminary evidence of its safety *in vivo*. Collectively, these findings suggest that zuclopenthixol has great potential for the effective treatment of melanoma and MBM.

4. Discussion

The efficacy of treatments for MBM prior to the advent of targeted therapies and immunotherapies has been poor, with patients experiencing a median overall survival of just 4-5 months [22]. While immunotherapy and targeted therapies have improved treatment outcomes, the survival of patients with MBM is still unsatisfactory [5], highlighting the urgent need for more effective treatment strategies. A major obstacle to the effectiveness of treatment for MBM is the BBB, which restricts drug penetration into the brain. Drugs that are able to cross the BBB and effectively inhibit melanoma therefore have tremendous potential for MBM treatment [7]. Anti-psychotic drugs can penetrate the BBB and have been approved and extensively studied. Moreover, some have been shown to possess anti-cancer activities, including inhibitory effects on brain tumors [19,23,24]. In previous studies, zuclopenthixol showed inhibitory activity against melanoma and other cancer types, prompting further exploration of its potential for the treatment of MBM [20,21,25]. The present results demonstrate that zuclopenthixol can effectively suppress the proliferation of various melanoma cell lines at low concentrations, resulting in G0/G1 cell cycle arrest and mitochondria-mediated intrinsic apoptosis. Furthermore, zuclopenthixol showed notable efficacy in inhibiting melanoma growth and brain metastasis in mice. These findings indicate that zuclopenthixol holds considerable promise in the treatment of MBM.

The present investigation affirmed that zuclopenthixol induces G0/G1 arrest in melanoma cells. The success of CDK4/6 inhibitors in breast cancer treatment suggests that targeting of the cell cycle may also prove effective in the treatment of melanoma. Numerous proteins participate in regulating the cell cycle, including cyclin-CDK complexes. Cyclin D binds to CDK4 and CDK6 to induce E2F-dependent transcription, DNA replication, increases in cyclin E and CDK2 levels, and subsequent phosphorylation of Rb, thereby propelling cancer cells through the G1/S checkpoint [26,27]. p27 acts as a pivotal inhibitor during the





Fig. 5. Zuclopenthixol induces apoptosis of melanoma cells via the mitochondrial-mediated intrinsic pathway. (A,B) Following treatment with various concentrations of zuclopenthixol for 24 h, cells were stained with Rh123 or dichloro-dihydro-fluorescein diacetate (DCFH-DA). FCM analysis was then performed to assess the decrease in the mitochondrial membrane potential ($\Delta\Psi$ m) and the reactive oxygen species (ROS) level within cells. (C) A375 cells were exposed to 20 µM zuclopenthixol, 15 mM γ -l-glutamyl-l-cysteinyl-glycine (GSH), or a combination of both for 24 h. The percentage of cells undergoing apoptosis was assessed using FCM. *p < 0.05, **p < 0.01, and ***p < 0.001, compared to the vehicle group.

G1/S cell cycle transition, and its elevated expression impedes the function of cyclin-CDK complexes [28]. The current results indicate that zuclopenthixol reduces the expression of CDK2, CDK4 and cyclin D1, while enhancing p27 expression. This implies that zuclopenthixol may induce G0/G1 cell cycle arrest by disrupting the relevant cyclin-CDK complexes. However, further experiments are needed to determine whether this is a direct or indirect effect.

The initiation of apoptosis is a promising strategy for cancer treatment. Currently, apoptotic pathways are primarily classified into two types. Extrinsic apoptosis is triggered when death ligands bind to membrane receptors, especially death receptors. This initiates the extrinsic apoptosis pathway, which is accompanied by activation of caspase 8 [29]. The other type is referred to as intrinsic apoptosis and is mediated by mitochondria. In this process, the integrity of the mitochondrial membrane is compro-



Fig. 6. Zuclopenthixol triggers protective autophagy and inhibits autophagy flow in melanoma cells. (A) The expression of LC3 and p62 in B16 and A375 cells was assessed after 24 h treatment with zuclopenthixol. (B) Representative images showing A375 cells transfected with an LC3B plasmid labeled with RFP-GFP in a tandem arrangement after being treated with zuclopenthixol (scale bar = 20 µm). (C) Viability of A375 cells treated with zuclopenthixol (7.5 µM) with or without chloroquine (CQ) (5 µM) or 3-methyladenine (3-MA) (1.5 mM). (D) Viability of B16 cells treated with zuclopenthixol (5 µM) with or without CQ (7.5 µM) or 3-MA (2 mM). (E) Rate of programmed cell death in A375 cells following exposure to zuclopenthixol (7.5 µM) alone or in conjunction with CQ (5 µM) or 3-MA (2 mM) (5 µM) alone or in combination with CQ (7.5 µM) or 3-MA (2 mM) for 48 h. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the vehicle group. GFP-LC3B, green fluorescent protein- microtubule associated protein 1 light chain 3 beta; 7-AAD, 7-aminoactinomycin D.

mised, thereby reducing $\Delta \Psi m$. Cytochrome c is subsequently released from the mitochondria into the cytoplasm, concomitant with the activation of caspase 9 [30,31]. In the present study, treatment with zuclopenthixol increased

the intracellular ROS level. Since tumor cells have heightened stress levels [32], this phenomenon can induce structural damage to mitochondria, compromise mitochondrial function, and subsequently trigger apoptosis. The partial



Fig. 7. *In vivo* efficacy of zuclopenthixol for the suppression of melanoma growth and brain metastasis. (A) Upon reaching a mean tumor size of approximately 100 mm³, mice with implanted human A375 cells were randomized into two groups (7 mice per group) and administered either the vehicle or zuclopenthixol (40 mg/kg). Tumor size and mouse body weight were recorded every three days. (B) Following 24 h treatment with varying levels of zuclopenthixol, B16 cells that successfully migrated through a transwell were stained with 0.5% crystal violet and counted. The number of migrating cells was used to calculate the rate of inhibition associated with different zuclopenthixol concentrations (scale bar = 100 μ m). (C,D) A brain metastasis model was established by intracarotid artery injection of C57 BL/6J mice. Zuclopenthixol treatment was initiated four days later. The growth of tumors in the mouse brain was quantified using bioluminescence imaging (n = 10). Signal intensity was quantified and is shown in (D). (E) Survival rates of mice with brain metastasis in the different treatment groups. (F) Changes in mouse body weight during zuclopenthixol treatment. *p*-values for comparison between the treatment and vehicle groups are denoted as **p* < 0.05 and ***p* < 0.01.

reversal of zuclopenthixol-induced apoptosis by the antioxidant GSH lends support to the role of ROS in this process. Numerous members of the BCL-2 family participate in the regulation of intrinsic apoptosis, with proapoptotic Bax and anti-apoptotic Bcl-2 being critical factors [33]. Our study found that zuclopenthixol reduces Bcl-2 expression and increases the Bax level, indicating involvement of the mitochondrial-mediated intrinsic pathway in zuclopenthixol-mediated apoptosis.

Evaluation of the anti-cancer effect of drugs in experimental animals is a critical step in predicting clinical efficacy. One of the primary goals of this study was to assess the potential of zuclopenthixol in the treatment of MBM. This was investigated using an experimental brain metastasis model. Current methods for establishing brain metastasis in mice include stereotactic-guided intracranial injection, left ventricular injection, intra-carotid artery injection, and spontaneous brain metastasis models [34]. Each of these methods has advantages and disadvantages, as detailed below. Intracranial injection does not include any phase of the metastasis cascade other than secondary organ outgrowth. Left ventricular injection may also lead to extracranial metastasis and is technically challenging, while spontaneous metastasis models are time-consuming and inefficient [34]. Here, we successfully mastered the technique of intra-carotid artery injection, thereby allowing an extensive study of brain metastasis. This model allows investigation of tumor cell circulation, BBB crossing, extravasation, colonization, and outgrowth in the brain. It does have some drawbacks, however, including its technical difficulty and the inability to study the tumor cell invasion process from tissue to blood vessels. Nevertheless, we observed that zuclopenthixol inhibited MBM more strongly than it inhibited subcutaneous tumors. A previous study found the concentration of fluphenazine in the mouse brain was much higher than in the plasma [35]. Since fluphenazine and zuclopenthixol are both anti-psychotic drugs that act in the brain, we speculate that the favorable therapeutic effect of zuclopenthixol on brain metastasis may be due to its high concentration in the brain.

Despite the promising results presented in this study, further work is required to advance the use of zuclopenthixol or its derivatives as effective anti-MBM drugs. The primary focus should be on elucidating the targets and the specific mechanisms of action. Previous studies demonstrated that anti-psychotic drugs can exhibit anticancer effects through various mechanisms. Liu et al. [19] reported that fluphenazine and trifluoperazine could inhibit the growth and migration of breast cancer cells by suppressing DRD2. Two other studies also highlighted the significant role of DRD2 in the anti-cancer activity of anti-psychotic drugs, including its inhibition of breast cancer cell self-renewal [36], and its inhibition of stressinduced malignant tumor progression [37]. Given that zuclopenthixol is a dopamine receptor antagonist used to treat schizophrenia, DRD2 could serve as a target for the anticancer activity of zuclopenthixol [20]. Interestingly, in the present study we noted that DRD2 expression in A375 cells decreased following zuclopenthixol treatment, indicating a possible effect on DRD2 expression. Further studies are required to examine the effect of zuclopenthixol on DRD2 expression in diverse melanoma cells, and to unravel the functions and mechanisms of DRD2 in zuclopenthixol-induced melanoma inhibition. The pivotal role of autophagy in

the anti-cancer activity of anti-psychotics has recently attracted significant attention. The current study, together with our previous work and research conducted by Lima et al. [38], collectively suggest that a viable strategy for treating melanoma may be to regulate autophagy. Lima et al. [38] found that TXA1, a thioxanthone compound, reduced the viability of melanoma cells by modulating autophagy, thus indicating its potential as a lead compound in the development of autophagy regulators with anti-cancer properties. Consistent with our present study and with prior research, zuclopenthixol is able to impede autophagy flux. However, the treatment of melanoma with zuclopenthixol and trifluoperazine gives rise to distinct outcomes with regard to autophagy. Zuclopenthixol induces protective autophagy in melanoma cells, so the suppression of autophagy could potentially amplify its inhibitory effects. On the other hand, treatment of melanoma cells with the anti-psychotic drug trifluoperazine is likely to induce cytotoxic autophagy, as evidenced by the partial reversal of its cytotoxic effect with 3-MA [21]. Moreover, another study reported that trifluoperazine could inhibit brain metastasis by blocking autophagic flux [39].

Several other studies have explored the anti-cancer activity and mechanism of zuclopenthixol. This drug was discovered as a promising compound that mimics moesin and has the ability to attach directly to the juxta-membrane area of HER2. The attachment specifically hinders the activation of HER2 in HER2-positive breast cancer, and effectively slows the progression of HER2-positive brain tumors in animal models [25]. Another study that screened a library of 3398 FDA-approved drugs found that zuclopenthixol inhibited the proliferation and migration of pancreatic stellate cells and disrupted tumor-stromal interactions, thus attenuating pancreatic cancer growth [40]. Zuclopenthixol was also reported to be a potential regulator of N6-methyladenosine in esophageal cancer [20]. However, the specific targets through which zuclopenthixol exerts its anti-cancer effects in melanoma and MBM remain unclear. Identifying these targets should contribute towards the development of novel anti-cancer therapies.

Combination therapy has become a common and effective clinical approach for treating tumors. It can enhance treatment efficacy, reduce toxicity by lowering drug doses, and also mitigate or delay the development of drug resistance. Current treatment options for MBM include immunotherapy, BRAF-targeted therapy, surgical intervention, stereotactic radiosurgery, and whole-brain radiation therapy. The use of combination therapy for the management of MBM has demonstrated promising outcomes, particularly for the combination of anti-CTLA-4 and anti-PD-1 immunotherapies, and the combination of immunotherapy with radiation therapy [41]. In the current study, autophagy inhibitors were found to synergistically enhance the anti-melanoma activity of zuclopenthixol. Therefore, combining zuclopenthixol with autophagy inhibitors, especially approved anti-tumor drugs that inhibit autophagy, may achieve better therapeutic effects against melanoma and MBM. Given the success of combination therapies in MBM treatment, future studies should investigate the efficacy of combining zuclopenthixol with existing treatment approaches.

Zuclopenthixol and trifluoperazine are potential drugs for the treatment of melanoma and MBM. They were discovered by our group using a drug repurposing strategy and during the same screening process [21]. Overall, zuclopenthixol exhibits better in vitro anti-proliferative activity than trifluoperazine, although they are both within the same order of magnitude. They also share several mechanisms of action, such as inducing G0/G1 cell cycle arrest and mitochondria-mediated intrinsic apoptosis. In addition, both can induce autophagy in melanoma cells and disrupt autophagic flux, although the effects of induced autophagy differ. Trifluoperazine induces potentially cytotoxic autophagy, whereas zuclopenthixol induces cytoprotective autophagy. The current study also employed additional experimental approaches to demonstrate the effects of zuclopenthixol. For example, we used the antioxidant GSH, whereas studies on trifluoperazine used NAC. This study also utilized the GFP-RFP-LC3 plasmid to study autophagic flux. Zuclopenthixol and trifluoperazine have different chemical structures, thus providing a better source for drug repurposing. In summary, the present study and previous research have shown that anti-psychotics are promising drugs for the treatment of melanoma and MBM. Further research is required to clarify their activities and mechanisms, thus allowing translation into clinically effective therapeutic drugs. Gaining a better understanding of the structureactivity relationships of zuclopenthixol and trifluoperazine, as well as identification of their targets, should facilitate the development of more effective drugs for the treatment of MBM.

5. Conclusions

Zuclopenthixol is a promising agent for the treatment of MBM due to its demonstrated ability to cross the BBB and exert potent anti-cancer effects. The findings in this study strongly support further research and development of zuclopenthixol as a potential therapeutic agent for MBM, with the ultimate goal of improving patient outcomes and quality of life for those affected by this devastating condition.

Availability of Data and Materials

Data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JZ designed the research study. WL, YX and AH performed the research. WL and SC analyzed the data. WL wrote the manuscript. All authors contributed to editorial

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changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal experiment was approved by the Ethics Committee of Sichuan University (No. 2021030544).

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Conflict of Interest

The authors declare no conflict of interest.

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