Universitat de les Illes Balears

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DOPAMINE D2 RECEPTOR SILENCING IN GLIOMA CELLS USING CRISPR/CAS9. EFFECTS ON THE RESPONSE TO PHENOTHIAZINES

Antoni Thomas Barrilado Jackson

Degree in Biochemistry

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	Yes	No	Yes	No
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Abstract

Glioblastoma multiforme is one of the main brain tumors and is characterized by its high aggressiveness and invasive capacity. Moreover, treatment is poor and patients diagnosed with this disease have a short life expectancy. Therefore, many efforts are being made in order to find new ways to treat this disease. **Phenothiazines** are a group of antipsychotic drugs whose main target is the **dopamine D2 receptor** (DRD2). Antitumor activities of these drugs have been described recently, although the exact mechanism is still unclear. For this reason, this project proposes the use of **CRISPR/Cas9** genome editing tool to generate a DRD2-deficient glioma cell line, evaluate the effect of phenothiazines in these cells and thus elucidate the mechanism of action. This would give rise to focus research on these drugs aiming their incorporation in future treatments against glioblastoma multiforme.

Resumen

El **glioblastoma multiforme** es uno de los principales tumores cerebrales y se caracteriza por su elevada agresividad y capacidad invasora. Además, el tratamiento suele ser poco eficaz y los pacientes diagnosticados con esta enfermedad tienen una esperanza de vida corta. Por ello, muchos esfuerzos se están haciendo para encontrar nuevas formas de tratar esta enfermedad. Las **fenotiazinas** son un grupo de fármacos antipsicóticos cuya principal diana es el **receptor de dopamina D2** (DRD2). Recientemente se han descrito actividades antitumorales por parte de estos fármacos, aunque el mecanismo exacto aún no está claro. Por ello, en este trabajo se propone el uso de la técnica de edición genómica **CRISPR/Cas9** para generar una línea celular de glioma deficiente en DRD2, evaluar el efecto de las fenotiazinas en estas células y así esclarecer el mecanismo de acción. Esto daría píe a centrar la investigación en estos fármacos con el objetivo de poder incorporarlos en futuros tratamientos contra el glioblastoma multiforme.

Abbreviations list

GBM	*Glioblastoma multiforme*
TMZ	*Temozolomide*
MGMT	*Methylguanine Methyltransferase*
CRISPR	*Clustered regularly interspace short palindromic repeats*
Cas	*CRISPR-associated protein*
crRNA	*CRISPR RNA*
tracrRNA	*Trans-activating crRNA*
PAM	*Protospacer-adjacent motif*
sgRNA	*Single guide RNA*
DSB	*Double strand break*
HDR	*Homologous directed repair*
NHEJ	*Non-homologous end joining*
DRD2	*Dopamine D2 receptor*
KO	*Knockout cells*
DMEM	*Dulbecco's Modified Eagle's Medium*
PBS	*Phosphate buffered saline*
BSA	*Bovine serum albumin*
TBS-T	*Tris buffered saline with Tween*
WT	*Wild-type cells*
PC	*Polyclonal cells*
MC	*Monoclonal cells*
qRT-PCR	*Quantitative reverse transcription-polymerase chain reaction*
TRD	*Thioridazine*
CPZ	*Chlorpromazine*
IC_{50}	*Inhibitory concentration*
PI	*Propidium iodide*
PS	*Phosphatidyl serine*
CaM	*Calmodulin*
EGFR	*Epidermal growth factor receptor*
GSC	*Glioma stem cell*

Index

Abstract ... 1
Resumen ... 1
Abbreviations list ... 2
Introduction ... 4
Glioblastoma multiforme ... 4
CRISPR/Cas9 ... 5
Phenothiazines ... 9
Objectives ... 10
Materials and methods ... 11
Cell lines and culture conditions ... 11
Transfection and CRISPR/Cas9 gene silencing ... 11
Protein isolation and quantification ... 12
Western Blot ... 12
Results ... 13
Experimental design ... 15
Western Blot Optimization ... 15
Quantitative Reverse Transcription-Polymerase Chain Reaction ... 15
Evaluation of cellular response to phenothiazines ... 16
Viability assay ... 17
Cell cycle analysis ... 18
Apoptosis assay ... 18
Discussion ... 19
Results from Experimental Work ... 19
Experimental design ... 20
Further directions ... 22
Conclusions ... 23
Bibliography ... 24

Introduction

Glioblastoma multiforme

Glioblastoma multiforme (GBM) is one of the most common and aggressive malignant brain tumors. In the United States, its annual incidence is around 3 per 100.000 people and has a median diagnosis age of 64 years, being men more prone to be affected. In addition, GBM has a poor prognosis with a 5-year survival rate of only 5 %. It accounts for 54,4 % of all malignant gliomas, tumors that originate from glial cells, which play a supporting role in the central nervous system and include astrocytes and oligodendrocytes. According to the World Health Organization, GBM is classified as a grade IV glioma, the highest grade based on factors such as mitotic activity, necrosis, vascular proliferation and invasiveness [1], [2].

GBM is divided into two main clinical types, primary and secondary. Primary GBM is the most common form and affects older patients as opposed to secondary GBM, which constitutes only 5 % of the cases and affects younger patients. Moreover, primary GBM suddenly manifests in a short time period while secondary GBM develops from a preexisting astrocytoma and has better prognosis [2], [3].

Further understanding of the genetic alterations and expression profiles has allowed the classification of this tumor into four different molecular subclasses: classical, mesenchymal, proneural and neural [3], [4]. Some of the molecular biomarkers that characterize these subtypes are:

- In classical GBM, most cases present loss of heterozygosity on chromosome 10 and chromosome 7 amplifications, *EFGR* amplification and deletions in *CDKN2A*, which encodes for *p16INK4A* and *p14ARF*. No alterations are found in *TP53, NF1, PDGFRA* or *IDH1* [4].
- Mesenchymal subclass is characterized by mutations in *PTEN* and *NF1*, expression of mesenchymal markers and less *EGFR* amplification [4].
- Proneural GBM is defined by *PDFGRA* aberrations, *TP53* and *IDH1* mutations and expression of oligodendrocyte development genes. Secondary GBM is frequently classified as proneural subtype [4].

- Neural subtype is characterized by a gene expression pattern similar to the one seen in normal brain tissue. Expression of astrocyte and oligodendrocyte markers is also observed, suggesting a more differentiated phenotype [4]. However, recent studies suggest that this subtype is non-tumor specific and only classifies GBM into classical, mesenchymal and proneural [5].

Therefore, the existence of different GBM subtypes requires the development of innovative personalized therapies that could better deal with this disease, but further investigation needs to be conducted to achieve this goal [2]–[4].

On the other hand, GBM still remains as a terminal disease with a median survival of 12 – 15 months. Typical treatment consists of surgical removal followed by radiation therapy and administration of temozolomide (TMZ). TMZ is an alkylating agent that promotes the methylation of the O^6 position on guanine, causing DNA damage that leads to cell apoptosis. An important aspect to take into consideration is that many GBM develop resistance to TMZ. The main mechanism of resistance involves methylguanine methyltransferase (MGMT) promoter methylation. MGMT is a DNA repair enzyme capable of correcting the change in guanine that TMZ produces, thus limiting the chemotherapeutic effect. When promoter is methylated, MGMT expression is reduced and consequently TMZ efficacy increases, but this is only observed in approximately 50 % of cases [6]. Moreover, despite aggressive treatment, recurrence is common due to the invasiveness capacity of the tumor. For this reason, more efforts are required to find new targets and therapeutic regimens to improve treatment against this disease [2].

CRISPR/Cas9

Clustered regularly interspace short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins form an adaptive immune system found in bacteria and archaea that has become one of the most important discoveries in recent years, revolutionizing the fields of biotechnology and genetic engineering [7]. Specifically, CRISPR/Cas9 system has been developed into an outstanding genome editing tool which has great potential in both basic and clinical research. In the future it could be even used to treat some diseases such as genetic disorders [8].

The CRISPR/Cas locus (**Fig. 1a**) is found in approximately 40 % and 90 % of bacterial and archaea genomes, respectively. It consists in an array of repetitive sequences where in between short fragments of DNA from invading pathogens called “spacers” are stored (CRISPR array) and a set of Cas genes that encodes for proteins with DNA cleavage activity [9].

There are different CRISPR systems depending on the variety of Cas genes and the organization of the protein complex that cleaves DNA in the process called interference. These systems are classified into two groups which are then subdivided into 6 types and several subtypes. In class 1 systems we found multi-Cas protein complexes and includes types I, III and IV CRISPR/Cas. Class 2 system is defined by one single Cas protein and is divided into types II, V and VI [7], [10].

This immune system acts in three phases: adaptation, CRISPR RNA (crRNA) expression and interference (**Fig. 1**).

- Adaptation is a complex multistage process (**Fig. 1b**). First, Cas1 and 2 identify the new foreign DNA and recognize a short sequence of 30 – 40 bp called “protospacer”, the precursor of the spacers found in the CRISPR array. With the endonuclease activity of the Cas1-2 complex, protospacer is extracted and then inserted between the leader sequence and the first repeat of the CRISPR array. This is how immunological memory is achieved.
- CRIPSR array is then transcribed into a precursor RNA which will be processed into several crRNA, each one containing the sequence of one specific spacer. These crRNAs are later coupled with different Cas proteins, depending on the type of CRISPR system, forming the crRNA-Cas complex (**Fig. 1c**) [9]. In type II CRISPR/Cas, crRNA maturation requires a small RNA called *trans*-activating crRNA (tracrRNA) that bounds to the pre-crRNA and then forms part of the mature crRNA-Cas complex. The tracrRNA is encoded upstream the Cas operon [10].
- Finally, interference consists in the degradation of foreign nucleic acid molecules that comes from reinfection (**Fig. 1d**). With this purpose, crRNA-Cas complex hybridize with the targeted DNA through complementary base pairing with crRNA and cleavage proceeds [9]. In types I, II and V systems, the complex needs to recognize a

protospacer-adjacent motif (PAM) next to the complementary sequence to execute cleavage [10].

Type II CRISPR/Cas mechanism is the simplest among the other types, only being necessary three components for DNA targeting and degradation: Cas9 protein, crRNA and tracrRNA. However, in 2012, a CRISPR/Cas9 system that combined tracrRNA and crRNA into a single guide RNA (sgRNA) was developed by Doudna and Charpentier, and proved to be as effective as the native system. This achievement made possible the use of this system as a simpler and more effective tool for genome engineering [11].

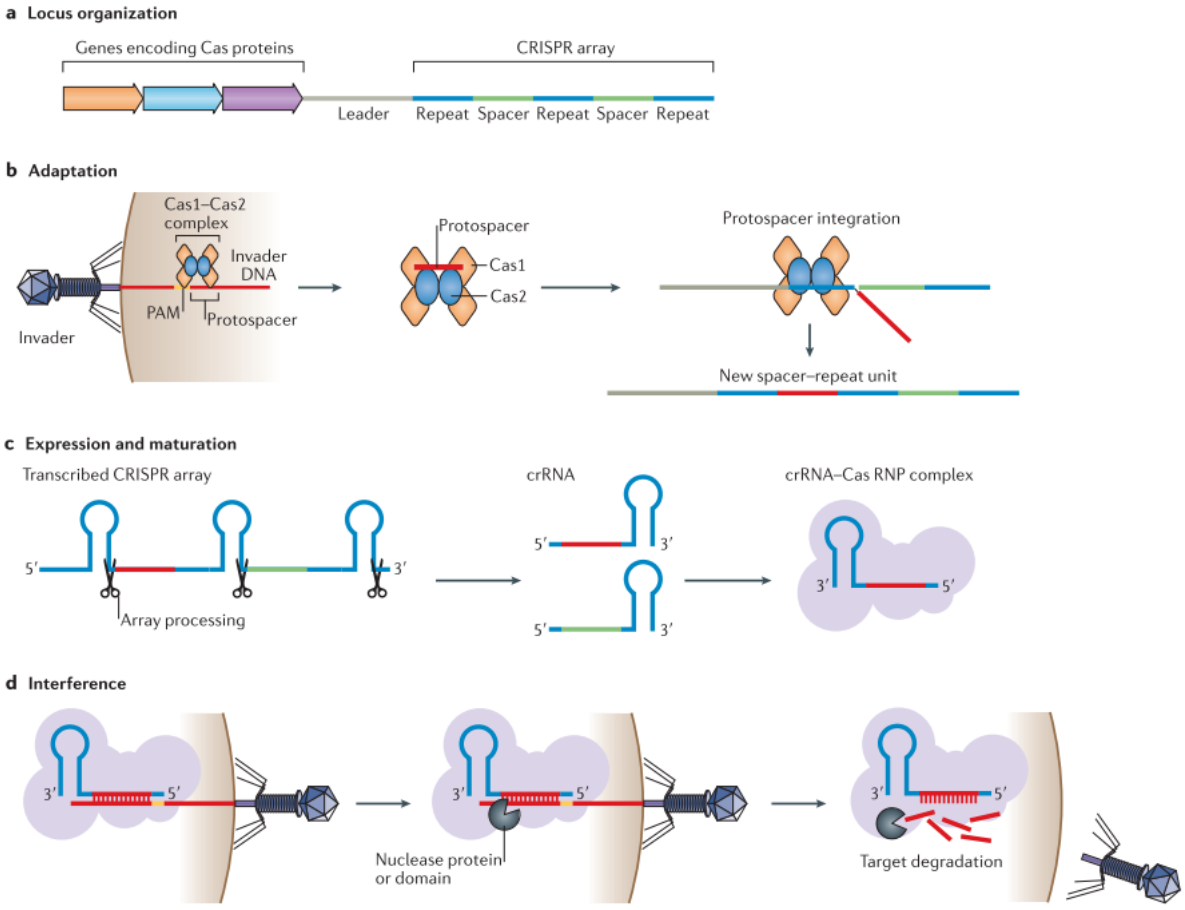

Fig. 1. CRISP-Cas adaptive immune system. (a) Basic structure of a CRIPSR-Cas locus. **(b,c,d)** CRISPR/Cas system acts in three stages: adaptation **(b)**, expression and maturation of crRNA-Cas complex **(c)** and interference **(d)**. Extracted from [9].

In order to conduct genome editing, it is required to design a sgRNA that is complementary to the sequence to be modified, which has to be right next to a PAM. Then, both sgRNA and Cas9 must be delivered to target cells using one of the multiple strategies available (for instance, a

CRISPR/Cas9 plasmid encoding Cas9 protein and sgRNA through virus) [12]. Upon delivery, CRIPSR/Cas9 technology produces site-specific double strand breaks (DSB) in target DNA using the Cas9 protein. Cas9-generated DSB are repaired by two mechanisms present in the cell: homologous directed repair (HDR) and non-homologous end joining (NHEJ) (**Fig. 2**). If a homologous template is provided, such as a plasmid, the DSB is repaired by HDR allowing precise sequence modifications like gene insertions or corrections. Otherwise, broken ends are ligated by NHEJ resulting in the introduction of small insertion or deletion mutations that can disrupt the expression of the targeted gene [13].

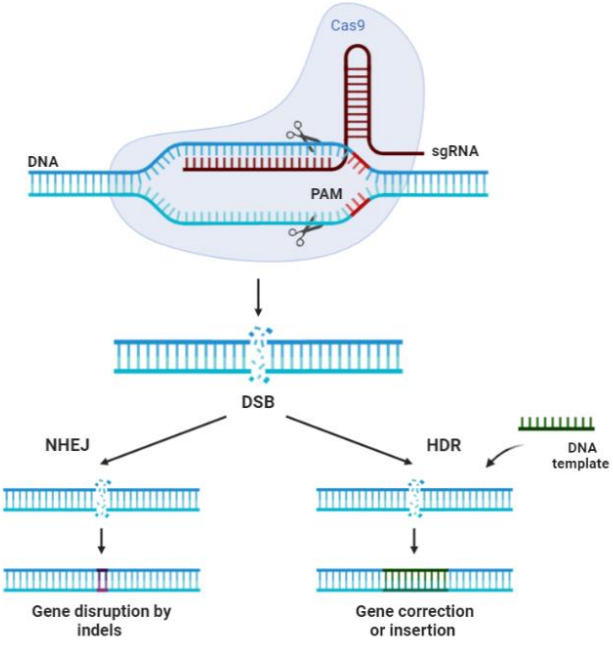

Fig. 2. Genome editing with CRISPR/Cas9. Created with Biorender.com based on [12].

CRISPR/Cas9 has great potential and many applications. Firstly, it can be used to study gene function or its implication in certain diseases by generating knockout models. Therefore, it has special relevance in the understanding of genetic disorders such as cancer o hereditary diseases. Many researchers are investigating the use of CRIPSR-Cas9 to treat these types of diseases by correcting mutations. However, one of the most important limitations is the possibility to produce off-target modifications that could be as harmful as the one being corrected. Moreover, the generation of more suitable models benefits pharmacological studies focused on discovering novel targets. Finally, other future possible applications are curing viral infections or modifying plant genomes to acquire disease resistance and improve crops [8], [14].

Phenothiazines

Phenothiazines are a group of drugs used in the treatment against psychiatric disorders such as schizophrenia. Their main structure consists in a tricyclic phenothiazine ring where substituents attached to C2 and N10 determine different types of phenothiazines and their specific properties. They are lipophilic compounds that cross the blood-brain barrier and block dopaminergic receptors due to the similarities in structure with dopamine (**Fig. 3**), producing the neuroleptic action [15].

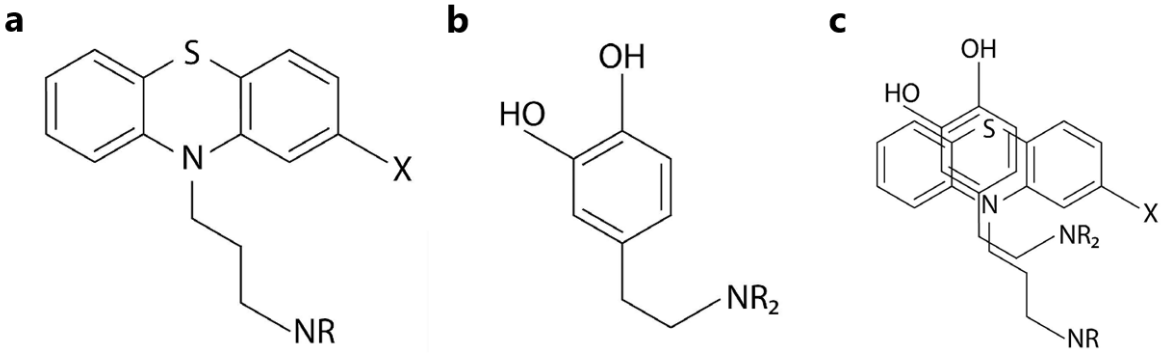

Fig. 3. Comparison of chemical structures between phenothiazine and dopamine. (a) Phenothiazine structure. (b) Dopamine structure. (c) Superposition of phenothiazine and dopamine structures. Adapted from [15].

Dopamine receptors are G protein-coupled receptors predominantly found in brain innervations involved in central nervous system functions such as voluntary movement, reward and learning. They are divided in two main groups, D1- and D2-class receptors, depending in their ability to modulate adenylyl cyclase activity. D1-class dopamine receptors include D1 and D5 receptors and usually activate adenylyl cyclase, whereas D2-class dopamine receptors (formed by D2, D3 and D4 receptors) induce adenylyl cyclase inhibition [16]. Of all, dopamine D2 receptor (DRD2) is the most important target of phenothiazines for their antipsychotic activity, showing more affinity to these compounds [17].

Despite phenothiazines being widely used as antipsychotic drugs since the 1950s, antiproliferative functions have been described in the last few decades, drawing special attention to their use in cancer treatment [17]. Some of these functions in GBM cells include cell cycle arrest, DNA damage and increased autophagy and apoptosis [18]–[21]. Importantly, phenothiazines are clinically approved drugs with the ability to cross the blood-brain barrier

and with a favorable brain distribution, which is one of the most important limitations in brain pharmacology. Therefore, their quick implementation into clinical trials in oncology could be possible [22]. In fact, in December 2019 a phase II clinical trial (NCT04224441) has begun to recruit patients to evaluate the effect of chlorpromazine, a type of phenothiazine, in combination with TMZ in GBM treatment [23].

The mechanism underlying the plausible antitumor effect of phenothiazines is not completely clear, and it is likely to implicate different pathways [20], [21]. Some studies have reported that DRD2 is linked to cell proliferation in different types of cancer [24]. These works described that blocking dopamine signaling with DRD2 antagonists reduces proliferation in GBM cell lines [25], [26]. Also, DRD2 silencing decreases by 70 – 90 % the growth of GBM cells [27]. Therefore, phenothiazines may act through the inhibition of the target DRD2 in GBM. In contrast, other studies suggest that the antitumor activity of phenothiazines would be through inhibition of other targets, such as calmodulin [15], [28]. Given these divergent points, there is a need to further study the mechanism of action of phenothiazines in glioblastoma. This would lead to the identification of the key targets that are involved and provide more effective therapeutic interventions.

Objectives

The main objective of the present project is to elucidate and clarify the role of DRD2 in the antitumor effects of phenothiazines. This knowledge would allow considering DRD2 as a potential therapeutic target. If the impact of DRD2 on antipsychotic treatments is negligible in the context of GBM, it would give rise to study alternative and potential pathways related to the mechanisms of action of phenothiazines. To do this, the following aims have been proposed:

1. Use Cas9-expressing glioma cells to generate DRD2 knockout (KO) cells through transfection of sgRNA.
2. Select and amplify the obtained DRD2 KO cells.
3. Characterize DRD2 KO cells.
4. Evaluate the cellular response of the DRD2 KO cells against antipsychotic phenothiazines.

Materials and methods

Cell lines and culture conditions

LN229 human glioblastoma cell line was established in 1979 from a right-frontal parieto-occipital glioblastoma. Cells present mutated *TP53* and possible homozygous deletions in *p16INK4A* and *p14ARF* tumour suppression genes. *PTEN* gene is unaltered [29].

- Cas9-expressing LN229 cell line was established by Miguel Garcia as part of his *Treball Fi de Grau* [30]. Briefly, wild-type LN229 cells were transfected with Cas9 plasmid using Lipofectamine 2000 according to the manufacturer's protocol. Then, selection of transfected cells was carried out with puromycin, as the plasmid also contained the puromycin N-acetyl transferase enzyme gene which confers resistance to the antibiotic.

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % inactivated Foetal Bovine Serum and 1 % Penicillin and Streptomycin in a humidified incubator at 37 °C and 5 % CO_2 atmosphere.

This cell line has adherent growth properties [29]. Therefore, before confluence, cells were washed with Phosphate Buffered Saline (PBS) and passaged using trypsin every 2/3 days per week. All reagents were obtained from Sigma-Aldrich.

Transfection and CRISPR/Cas9 gene silencing

Between 2,5 – 3 · 10^5 Cas9 LN229 cells were seeded in each well of a 6-well plate 24 h before transfection in order to reach approximately 80 % confluence at the time of transfection.

One sgRNA targeting DRD2 gene (5'- *ACA GAU UCA GUG GAU CCA UC* -3') was designed using the Custom Alt-R® CRISPR-Cas9 guide RNA Design tool from Integrated DNA Technologies website (https://eu.idtdna.com/pages). Selection of sgRNA was based on the on- and off-target scores provided by the tool, which predict the editing capacity at the targeted sequence or at other undesired locations, respectively.

On the day of transfection, medium of the cells was replaced with DMEM without antibiotics. For transfection, 250 µl of Opti-MEM medium (Gibco) was mixed with 4 µL of Lipofectamine 2000 (Invitrogen) in a sterile Eppendorf tube. In a different tube with 250 µl Opti-MEM

medium, 25 or 50 nM sgRNA was added. After briefly mixing, both solutions were incubated for 5 min and Lipofectamine solution was added to the sgRNA solution. Upon mixing, the final solution was incubated at room temperature for 20 min to form sgRNA-Lipofectamine complexes and then added to the cells. After 72 – 96 h of incubation, cells were amplified and collected for Western Blot analysis.

To generate a monoclonal cell line from the polyclonal pool of stable cells described above, around 50-100 cells were seeded in a 100-mm dish. After two or three weeks, there were several colonies on the plate, each colony being a plausible monoclonal population. Next, colonies were picked and transferred to a 12-well plate. After culturing and expanding, clones were harvested and analysed by Western Blot to select the best clones, i.e. those with the lowest expression of DRD2. Generation of a monoclonal cell line results in a cell population that is more likely to retain effective targeted gene knockout.

Protein isolation and quantification

Cells were washed with PBS and lysed by scrapping in lysis buffer containing 50 mM pH 7,4 Tris, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100 and H_2O, supplemented with PhosSTOP™ phosphatase inhibitor cocktail (Roche) and cOmplete™ Mini protease inhibitor cocktail (Roche). Then samples were centrifuged at 14.000 x *g* for 20 min at 4 °C and protein quantification was carried out with DC Protein Assay (Bio-Rad), following manufacturer's instructions. This kit is a colorimetric test based in the Lowry assay, in which proteins cause the reduction of the Folin reagent, giving a product with maximum absorbance at 750 nm [31] which was measured with Biotek Powerwave HT Microplate Spectrophotometer. Sample protein quantification was assessed with a Bovine Serum Albumin (BSA) standard.

Western Blot

After protein quantification, equal amounts of protein were prepared for Western Blot using Sample Buffer, containing 50 mM pH 6,8 Tris, 10 % Glycerol, 2,5 % SDS, 5 % β-Mercaptoethanol, Bromophenol blue and H_2O. As the molecular weight of DRD2 is reported to be approximately 50 kDa [32], between 20 – 60 µg of sample protein were loaded onto 10 % SDS-polyacrylamide gel for electrophoresis. Electrophoresis program consisted in 80 V for sample stacking and 110 V for resolving. Then, samples were transferred to nitrocellulose membranes using wet transfer at 90 V for 1 h. Next, membrane was immersed in Ponceau

staining solution for 5 min with gentle agitation to monitor the efficiency of transfer to the membrane. For staining removal, membranes were washed with PBS.

Subsequently, membrane was blocked with 5 % skimmed milk powder in Tris Buffered Saline with 0,05 % Tween (TBS-T) for 1 h, followed by 3 washes with TBS-T for 5 min each. Then, membrane was incubated overnight with a 1:1.000 dilution of Rabbit Anti-DRD2 Polyclonal Antibody (Millipore AB5084P) in TBS-T with 5 % BSA at 4 °C. Then, membrane was washed again 3 times with TBS-T and incubated with anti-rabbit secondary antibody (Dako) (1:2.000-1:10.000) in TBS-T with 2 % skimmed milk powder for 1 h at room temperature with agitation. Finally, membrane was washed 3 times with TBS-T and one with TBS. Secondary antibody used was labelled with horse radish peroxidase which allowed chemiluminescent protein detection using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Also, Mouse Anti-CRISPR-Cas9 antibody (Abcam) was used to assess Cas9 expression. Tubulin was used as loading control and detected with Mouse Anti-α-Tubulin antibody (Sigma-Aldrich).

Results

Before starting with the transfection and silencing process, LN229 Cas9-expressing cells established by Miguel Garcia [30] were examined through Western Blot in order to: a) confirm Cas9 expression and b) select the optimal LN229 Cas9 cell line for the experimental purposes.

As expected, the three different LN229 Cas9 cell lines analyzed expressed Cas9, in contrast to wild-type (WT) cells (**Fig. 4**). Therefore, any of the cell lines could be used to generate DRD2 deficient cells. The **Cas9 2** cell line was chosen for that purpose.

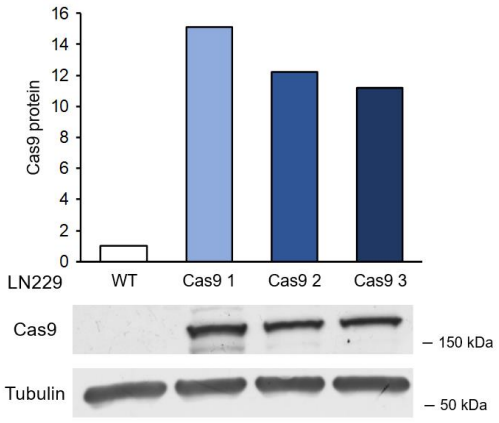

Fig. 4. Cas9 expression in LN229 Cas9-expressing cell lines. Western Blot analysis of Cas9 expression in three different LN229-Cas9 cell lines. Tubulin expression was used to normalize data.

Transfection of sgRNA targeting DRD2 was performed three times using different concentrations of sgRNA. CRISPR-Cas9 allows gene silencing and Western Blot analysis revealed that DRD2 expression was reduced in some of the transfected cell lines although not completely (**Fig. 5**). This could be explained by the presence of non-transfected cells that still expressed DRD2 in a polyclonal cell pool, in which efficacy of transfection and silencing differed between cells. Therefore, with the aim of isolating completely silenced cells and establishing a pure KO line, monoclonal cell lines were obtained from the cells transfected with 50 nM sgRNA targeting DRD2.

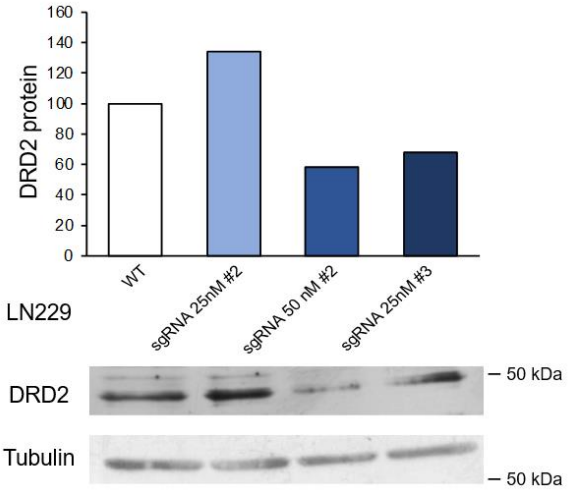

Fig. 5. DRD2 expression in transfected cells. Cells from the second (#2) and third transfection (#3) were analyzed. Tubulin expression was used to normalize data.

Five different monoclonal cell lines were obtained. However, several non-specific bands appeared in the Western Blot (**Fig. 6**) and DRD2 expression could not be assessed correctly. Moreover, polyclonal (PC) and monoclonal (MC) cell lines had similar bands around 50 kDa, suggesting that DRD2 knockout had not been effective or that cells had recovered DRD2 expression. Lower band intensity for MC 3 was due to the fact that less sample was loaded by error, confirmed with previous Ponceau staining. Regardless of this unexpected result, the following step of our experimental strategy was to repeat and improve the Western Blots but unfortunately, due to the COVID-19 crisis, the experimental procedure was ended at this point.

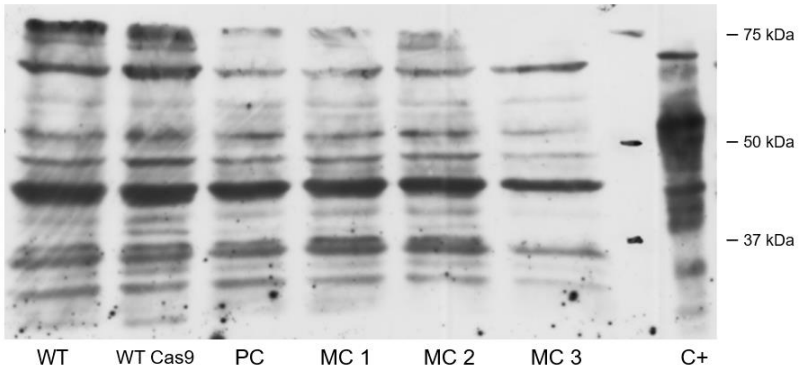

Fig. 6. Non-specific bands in Western Blot for DRD2 expression assessment. Three out of the five monoclonal (**MC**) cell lines were analyzed for DRD2 expression in this gel. Polyclonal (**PC**) cell line correspond to the cells that were transfected with 50 nM sgRNA. Positive control (**C+**) consisted in mouse hippocampus tissue lysate kindly provided by the Neuropharmacology Research Group (UIB).

Experimental design

As it was impossible to continue with the experimental work, I am going to explain the procedures that should be conducted to achieve the initial objectives.

Western Blot Optimization

Results from Western Blot were not appropriate to be analyzed and could not be considered as solid due to the high amount of unspecific bands that appeared in the detection with the anti-DRD2 antibody. Therefore, optimization is required.

DRD2 is a membrane protein so it may require a more aggressive protein extraction. With this purpose, Triton X-100 lysis buffer would be substituted with RIPA buffer and sample sonication could be considered [27]. Another variable which could be addressed is increasing the concentration of protein loaded in gels. For instance, protein could be concentrated by using Amicon® Ultra centrifugal filters [33]. Moreover, the use of more suitable positive controls would be helpful for knowing if there is any technical problem, such as antibody non-specificity.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Another option to assess DRD2 expression is by mRNA quantification through Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). This technique was planned to be performed together with Western Blot optimization. To do this, total RNA would be

extracted using TRIzol isolation reagent (Invitrogen) following manufacturer's instructions. Briefly, it consists in precipitating RNA from the aqueous phase with isopropanol, washing with 75 % ethanol and resuspending in RNAse-free water. Then, RNA would be quantified using Nanodrop 2000 (Thermo Fisher) set at 260 nm. Once quantified, 1 – 2 µg of total RNA would be reverse-transcripted to cDNA using High-Capacity RNA to cDNA™ kit (Thermo fisher) with the following conditions: 1 h at 37 °C and 5 min at 95 °C.

Subsequently, qRT-PCR would be carried out with SYBR Green technology on a Light Cycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel Switzerland) and DRD2 specific primers (Fw: 5'- *CAT CTT CGT CAC TCT GGA CG* -3', Rv: 5'- *AGT GAA CAG GCG GAG AAT GG* -3'). PCR program would consist of a preincubation step (5 min at 95 °C), followed by 45 cycles consisting of a denaturation step (10 s at 95 °C), an annealing step (10 s at 60 °C), and an extension step (10 s at 72 °C).

Each sample would be assayed in triplicate and quantified by delta-delta Ct method, normalizing with GAPDH (Fw: 5'- *TGC ACC ACC AAC TGC TTA GC* -3', Rv: 5'- *GGC ATG GAC TGT GGT CAT GAG* -3') or 18S expression (5'- *GTA ACC CGT TGA ACC CCA TT* -3', Rv: 5'- *CCA TCC AAT CGG TAG TAG CG* -3'). In each assay, a negative control lacking cDNA template would be analyzed.

Evaluation of cellular response to phenothiazines

Once DRD2 knockout in LN229 is completely assured, the next step would be evaluating the response to phenothiazines. I would have selected thioridazine (TRD) and chlorpromazine (CPZ) for evaluation based on the background information that supports these two phenothiazines as interesting candidates to study in cancer treatment.

It has been proved that TRD reduces GBM cell lines growth by cell cycle arrest and induces apoptosis and autophagy in a dose-dependent manner [19], [20]. TRD can also cause DNA fragmentation and enhance caspase activity [20]. Moreover, TRD combined with TMZ produced a synergistic increase of apoptosis, indicative of the potential therapeutic use of this phenothiazine [19]. In patients treated with TRD, plasma levels of this phenothiazine reached micromolar concentrations as opposed to the phenothiazine trifluoperazine, therefore TRD could be more effective in GBM treatment [34].

On the other hand, as mentioned before, CPZ is going to be tested in combination with TMZ in a phase II clinical trial (NCT04224441) [23]. Several activities have been described in cancer cells: cytotoxic activity, nuclear fragmentation, autophagy induction and inhibition of dopamine D2 and glutamate receptors [21]. In glioma U87 MG cells, CPZ induced autophagy that led to cell death through Akt/mTOR signaling pathway inhibition. In addition, the antiproliferative activity of CPZ would also be achieved through mitotic kinesin KSP/Eg5 inhibition or increased $p21^{Waf1/Cip1}$ expression, causing cell cycle arrest in G2/M phase [18].

Knowing this information, the assessment of TRD and CPZ treatment in DRD2 KO cells would consist in viability assays, followed by the analysis of cell cycle and apoptosis if possible. WT cells would also be subjected to the same conditions as DRD2 KO cells in order to compare both responses to treatment and elucidate if DRD2 plays a role in the anti-cancer properties of these phenothiazines.

Viability assay

Through viability assays and dose-response curves it is possible to know the half maximal inhibitory concentration (IC_{50}) of a certain drug, which is the concentration at which cell proliferation is reduced by 50 %. DRD2 deficiency may alter the IC_{50} of phenothiazines in DRD2 KO cells, suggesting that DRD2 is important for the antiproliferative activity of TRD and CPZ.

For this reason, viability of cells subjected to phenothiazine treatment would be assessed using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega). This kit is based on the luciferase reaction, which produces light in direct proportion to the concentration of ATP, which is produced by active cells. Therefore, through light detection it is possible to determine cell viability [35].

5.000 DRD2 KO or WT cells would be cultured in each well of a 96-well plate and treated with the following concentrations of phenothiazines, as previously performed in the laboratory [36]:

- For TRD and CPZ: Control (0 µM), 2,5 µM, 5 µM, 10 µM, 20 µM, 40 µM and 80 µM.

For each condition, there would be three replicates and they would be incubated for 48 h. Then, cells would be analyzed using the mentioned kit, following the manufacture's protocol.

Cell cycle analysis

Cell cycle analysis would be performed through flow cytometry to assess if CPZ and TRD can induce cell cycle arrest in DRD2 KO cells. First, approximately $1{,}5 \cdot 10^5$ cells would be cultured in 60-mm dishes. Three 60-mm dishes would be needed for each type of cell (WT or KO) and treatment (TRD or CPZ). Based on previous experiments of the laboratory (data not published), treatment conditions for both cell types would be:

- For TRD: Control (0 µM), 10 µM and 15 µM.
- For CPZ: Control (0 µM), 12,5 µM and 20 µM.

After 24 h of treatment, cells would be collected keeping the culture medium of the dish, which contains the apoptotic cells. Then, all cells would be centrifuged, washed with PBS and fixed with 70 % ethanol. Upon fixation, cells would be washed twice with PBS, treated with RNase and stained with propidium iodide (PI). Finally, cells would be analyzed with a flow cytometer. PI is a compound that intercalates in DNA and emits fluorescence in proportion to the quantity of DNA. This allows to differentiate the stage of the cell cycle as the amount of DNA varies within stages [37].

Apoptosis assay

Apoptosis assay would be conducted using Alexa Fluor® 488 annexin V/Dead Cell Apoptosis kit (Thermo Fisher) in order to determine if CPZ or TRD are able to induce apoptosis in DRD2 KO cells. This kit allows the detection of phosphatidyl serine (PS) when it translocates from the inner to the outer membrane, which is one of the biochemical changes that occur during apoptosis. It contains the anticoagulant annexin V, which is able to bind to the exposed PS on cell surface and is conjugated to the fluorophore Alexa Fluor® 488. Additionally, it includes PI which stains dead cells. Therefore, with Alexa Fluor® 488 and PI, apoptotic and dead cell populations can be analyzed through flow cytometry [38].

With this objective, $1{,}5 \cdot 10^5$ cells would be cultured in 60-mm dishes and treated for 48 h with the same concentrations of phenothiazines as described for the cell cycle analysis. Again, WT cells would also be treated and compared with DRD2 KO cells. Then, medium with dead cells would be saved and cells from plate would be harvested and added to the saved medium. After centrifugation and washing with cold PBS, cells would be stained with Alexa Fluor® 488

Annexin V and PI following manufacturer's instructions, and finally analyzed using a flow cytometer [38].

Discussion

Phenothiazines are a type of drugs used in psychiatric treatment against diseases like schizophrenia. The main activity consists in the inhibition of dopamine signaling by interacting with DRD2, one of the receptors for this neurotransmitter. However, at present time, several studies have described antitumor properties that make phenothiazines interesting candidates to use in GBM treatment [17]. In fact, some studies report that schizophrenic patients treated with phenothiazines have a lower incidence of GBM [26]. Most importantly, a phase II clinical trial (NCT04224441) has been initiated with the aim of evaluating the therapeutic potential of the combination of TMZ with CPZ for GBM treatment [23]. Despite this, the molecular mechanism behind the cytotoxic and cytostatic effects still remains to be clarified. The present project intended to evaluate the response to phenothiazines in DRD2 KO glioma cells obtained through CRISPR/Cas9 with the objective of addressing the above-mentioned matter. Unfortunately, due to technical problems and the COVID-19 pandemic, the project could not be concluded.

Results from Experimental Work

Although they are not reliable, results obtained from the last Western Blot were not as expected (**Fig. 6**). It raises concerns about the effectiveness of CRISPR/Cas9 gene silencing by showing no differences between the WT and the alleged DRD2 KO cells. Perhaps PC and MC cell lines recovered DRD2 expression or they were never silenced, despite the results observed in figure 5. Therefore, Western Blot must be improved and repeated to confirm the levels of DRD2. On the other hand, results show a high amount of bands detected, indicating that the anti-DRD2 antibody used may be unspecific and thus not practical. For that reason, qRT-PCR is suggested to be conducted in order to determine if DRD2 silencing was accomplished correctly. If that was not the case, the whole process would have to be repeated, but preferably making some changes that are discussed here.

First, DRD2 expression should be assessed in different glioma cell lines (i.e. U87 MG, LN229 and U251 MG) to select the best cell line to work with, the one which expresses DRD2 the

most. This would allow the observation of reliable differences in DRD2 expression between WT and DRD2 KO cells. Additionally, sgRNA may not be completely efficient, therefore, validating more than one sgRNA targeting DRD2 would ensure that at least one of the polyclonal pools of cells obtained is correctly silenced. On the other hand, solid and reliable Western Blot results that indicate the loss of DRD2 expression are crucial for continuing with the evaluation of the phenothiazine effect in cells. For this reason, Western Blot technique cannot be omitted and should be optimized. Searching for more suitable positive controls is one of the possibilities to assess, but one of the best options to consider would be establishing a glioma cell line which over-expressed DRD2.

However, if failure in generating DRD2 KO cells with CRISPR/Cas9 is recurrent and demonstrated, an alternative to continue with the project would be the downregulation of DRD2 by using short hairpin RNA or small interference RNA, as performed by Li et al. [27]. These RNA molecules are able to interfere with mRNA processing and reduce DRD2 expression [27].

Experimental design

The main objective of the proposed experimental design is to assess the antitumor effect of the phenothiazines CPZ and TRD in cells deficient for their main target, DRD2. Once conducted, it will be possible to elucidate the functional and specific role that DRD2 plays in the antitumor activity of these phenothiazines in GBM. Three different scenarios would be possible depending on the results obtained: a) DRD2 KO cells are resistant to phenothiazine treatment, b) DRD2 KO cells are affected in the same way as WT cells or c) phenothiazine treatment promotes cell death in DRD2 KO cells but at a lower level than in WT cells.

If DRD2 KO cells are not affected by phenothiazines, this would suggest that DRD2 is the main target for both antipsychotic and antitumor properties of these drugs. No changes in cell viability or apoptosis in comparison with WT cells would indicate that the mechanism by which phenothiazines act is through DRD2 inhibition. It has already been described that dopamine signaling contributes to glioblastoma proliferation [25], [39] and that DRD2 deficiency reduces growth in glioma cell lines [27]. Therefore, it is important to first compare the possible differences between WT and DRD2 KO cells due to disrupted DRD2 expression for then being able to distinguish the alterations in viability caused by the phenothiazine treatment.

However, this scenario is the least likely to occur due to the evidence from other studies which will be further discussed.

In the hypothetical case b), DRD2 KO cells would suffer the same consequences as WT cells while exposed to phenothiazines, despite the absence of DRD2. This would suggest that DRD2 is not involved in the antitumor properties of phenothiazines and, therefore, the existence of other targets that could explain these effects. Calmodulin (CaM) is a calcium binding protein which has an important role in intracellular signaling and the regulation of processes involved in proliferation, and is suggested to be a target of phenothiazines [15], [28], [40]. The chemical structure of these compounds enables the inhibitory interaction with CaM and the alteration of several enzyme activities that this protein regulates, including different kinases and phosphatases [15]. Kau et al. [28] demonstrated that FOXO1a, a forkhead family transcription factor which promotes cell cycle arrest and apoptosis, is retained in the nucleus and active in the presence of phenothiazines. However, they suggested that a CaM-dependent mechanism is behind this effect because with other dopamine receptor antagonists it was not observed [28]. Moreover, some studies have reported that the capacity of TDZ to induce autophagy and apoptosis is independent of DRD2 in glioma cell lines [20], [41]. In conclusion, if results indicate that DRD2 deficiency does not alter phenothiazine activity, it could support the idea of CaM being responsible for the antitumor effect and give importance to focus in this protein in further studies.

The last possible scenario implies that DRD2 inhibition is important for reducing cell proliferation but still other mechanisms, such as CaM inhibition, may also be involved in the molecular activity of phenothiazines. This would be assumed if DRD2 KO cells are less affected by phenothiazine treatment in comparison to WT cells. DRD2 signaling has been related to other signaling pathways like the Epidermal Growth Factor Receptor (EGFR) axis, which is commonly altered in glioblastoma [2], [27]. EGFR signaling enhances cell survival by activation of the MAPK pathway through Ras-GTP bounded with Raf-1. It has been described that DRD2 activates a GTPase that inhibits Rap1-GTP, which is capable to sequester Raf-1, therefore contributing to EGFR signaling [42]. Most importantly, the combination of EGFR inhibitors with the DRD2 antagonist called haloperidol, which in fact is another type of antipsychotic, resulted in a synergistic anti-glioblastoma effect, which could be considered for future treatment strategies [27]. A study in U87 MG glioblastoma cell line proved that DRD2 inhibitors, including

TDZ, are able to reduce spheroid formation in a DRD2-dependent manner [43]. Furthermore, other studies have observed the capacity of DRD2 antagonists to reduce proliferation in other types of cancer, such as colorectal and uterine cervical cancer cells [24], [44]. Therefore, if at the end of the proposed experimental design it is proved that DRD2 is an important target for the phenothiazine antitumor mechanism, along with the supporting information, more importance will be given to DRD2 and phenothiazines in GBM research.

Further directions

This project could be followed by further studies addressed to elucidate the specific mechanism by which phenothiazines promote cell death in glioblastoma. By this means, new targets for better treatment options could be discovered and exploited. It would be interesting to study in these DRD2 KO cells some of the specific alterations that have been attributed to phenothiazines in order to clarify if they are produced by the DRD2 inhibition. For example, FOXO1a nuclear localization could be assessed in order to first confirm if this phenothiazine effect is given in glioblastoma cells. Then, it could give support to the idea of CaM as a target of phenothiazines and regulator of FOXO1a as described previously [28]. Additionally, TRD and CPZ are also able to induce autophagy, a process that can lead to reduced cell proliferation and apoptosis. Autophagy intermediators, such as LC3 I and II, could be studied in DRD2 KO cells under phenothiazine treatment in order to seek for a connection between autophagy and the receptor [18], [19].

On the other hand, searching for new treatments is one of the principal objectives in GBM research nowadays [45]. In glioma cell lines, DRD2 antagonists caused a synergistic suppression of growth when combined with TMZ, the current drug used for chemotherapy [46]. Also, treatment with TRD has shown to enhance the effects caused by TMZ in glioblastoma [19]. Therefore, the combination of phenothiazines with other agents used in chemotherapy like TMZ may be an interesting strategy to consider for GBM treatment and should be studied [19], [46]. It is worth mentioning that one of the main reasons of GBM therapeutic resistance is the presence of undifferentiated cells with the ability of self-renewal termed Glioma Stem Cells (GSC) [2]. These cells have also been proposed as responsible for the invasiveness, immune evasion and proangiogenic capacity of GBM [47]. Most importantly, it has been observed that DRD2 is highly expressed in GSC and that it is important for the self-

renewal and propagation abilities of these cells [39]. In fact, TRD has proved to reduce cell viability and the expression of GSC markers in GBM sphere cells [20]. If the experimental design of this project is proved to be an effective and reliable way to study the antitumor mechanism of phenothiazines, it could be then applied in GSC to evaluate their response to these drugs.

For these reasons and the fact of already being clinically approved, phenothiazines are strong candidates to fulfill the urgent need for novel drugs in GBM treatment [22]. This project could encourage further studies on DRD2 and phenothiazines in order to seek for new therapeutic strategies to use in GBM.

Conclusions

This project was aimed to elucidate the role of DRD2 in the antitumor mechanism of phenothiazine antipsychotic drugs, but unfortunately it could not be concluded due to unexpected events. However, the proposed experimental procedure, using the revolutionary genome editing tool CRISPR/Cas9, still remains as an interesting way to assess this issue.

Moreover, background information provides considerable support to the use of phenothiazines in GBM treatment, but further investigation on these drugs and DRD2 needs to be conducted in order to understand the exact mechanism and discover new approaches to define alternative therapies.

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