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# Antipsychotic drugs elicit cytotoxicity in glioblastoma multiforme in a calcium-dependent, non-D$_2$ receptor-dependent, manner

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## Abstract

Dopamine D$_2$-like receptor antagonists have been suggested as being potential anticancer therapeutics with specific utility for central nervous system cancers due to their ability to cross the blood-brain barrier. Despite a plethora of data reporting anticancer effects for D$_2$R antagonists in cell or animal studies, the ligand concentrations or doses required to achieve such effects greatly exceed the levels known to cause high degrees of occupancy of the D$_2$ receptor. To resolve this conundrum, we interrogated a panel of glioblastoma multiforme (GBM) cell lines using D$_2$ antagonists of varying chemotype. We studied the cytotoxic effects of these compounds, and also ascertained the expression of D$_2$ receptors (D$_2$R) on these cells. Although several chemotypes of D$_2$R antagonists, including phenothiazines and phenylbutylpiperidines, were effective against GBM cell line cultures, the highly selective antagonist remoxipride had no anticancer activity at biologically relevant concentrations. Moreover the D$_2$R antagonist-induced cytotoxicity in monolayer cultures was independent of whether the cells expressed D$_2$R. Instead, cytotoxicity was associated with a rapid, high-magnitude calcium flux into the cytoplasm and mitochondria, which then induced depolarization and apoptosis. Blocking this flux protected the GBM cell lines U87MG, U251MG, and A172. Together, these data suggest that the cytotoxicity of these D$_2$R antagonists involves calcium signaling mechanisms, not D$_2$R antagonism. Repurposing of existing drugs should focus on the former, not latter, mechanism.

**KEYWORDS**
antipsychotic, calcium signaling, dopamine, glioblastoma

# 1 | INTRODUCTION

Glioblastoma multiforme (GBM) is a very lethal form of central nervous system (CNS) malignancy that is difficult to treat due to the relative impermeability of the blood brain barrier (BBB) that excludes many chemotherapeutic agents.[1] Repurposing compounds with known toxicity profiles, but which can cross the BBB, could theoretically lead to novel therapeutics.[2] One obvious group of candidates are drugs used in psychiatry (eg, antipsychotics, antidepressant, anxiolytics, etc) whose actions depend on CNS availability. Many of these compounds are approved by the Food and Drug Administration for the treatment of mental illnesses, including depression, anxiety, bipolar disorder, and schizophrenia. The antipsychotics may be of special interest due to their long history of use in humans, their well-characterized pharmacokinetic and pharmacodynamic profiles, their ability to concentrate in the brain to higher levels than in the periphery, and the sheer number and variety of chemical structures available. While all of the antipsychotics vary in structure and pharmacodynamic properties, they share the common mechanism of targeting $D_2$-like dopamine receptors, usually as antagonists.[3]

Recent research has suggested that a number of these approved dopamine D2 receptor (D2R) antagonists have anticancer efficacy and modest cytotoxic selectivity for cancer vs normal cells,[4,5] implicating $D_2$-like receptors as potential targets for anticancer therapeutics in numerous studies.[6-8] Although studies have shown anticancer effects for dopamine $D_2$-like receptor antagonists, ligand concentrations required for such effects exceed those pharmacologically predicted for high degrees of $D_2R$ occupancy (Figure 1B).[9] Thus, although the $D_2R$ has been the postulated target, it is unclear which of its signaling mechanisms are involved (eg, Akt signaling, lipid modulation, autophagy, etc), or even if the effects are actually due to interactions with the $D_2R$.[10] To understand the relationship between $D_2R$ antagonists and observed anticancer activity, we chose to interrogate a panel of glioblastoma multiforme (GBM) cell lines using $D_2R$ antagonists of varying chemotypes. GBM was selected as our focus since dopamine
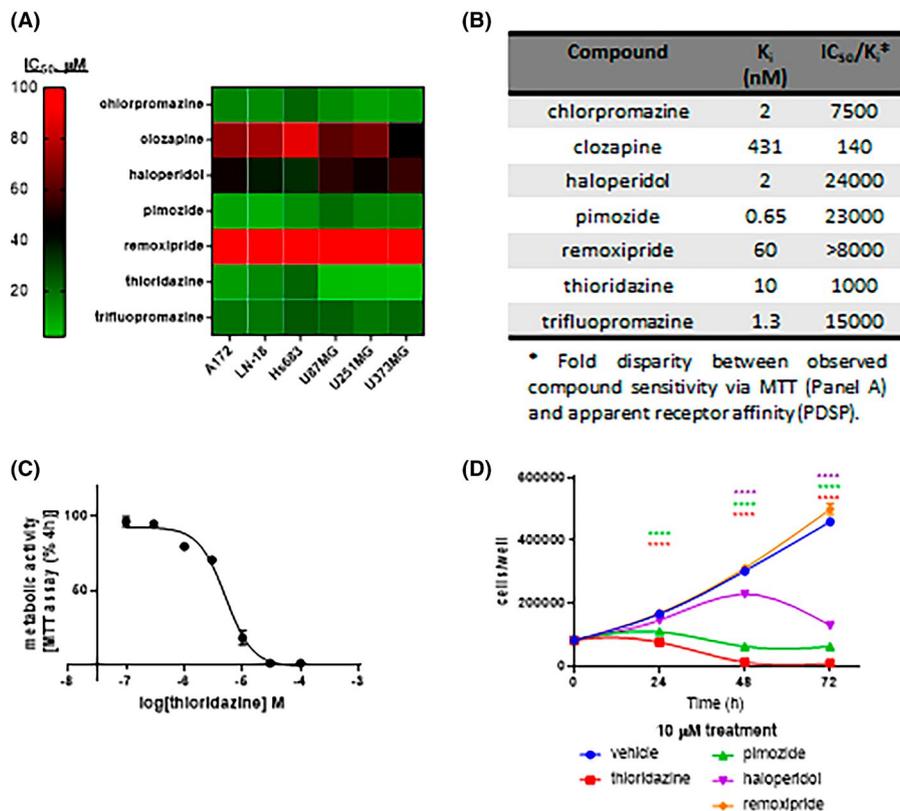


| Compound | $K_i$ (nM) | $IC_{50}/K_i$* |
|---|---|---|
| chlorpromazine | 2 | 7500 |
| clozapine | 431 | 140 |
| haloperidol | 2 | 24000 |
| pimozide | 0.65 | 23000 |
| remoxipride | 60 | >8000 |
| thioridazine | 10 | 1000 |
| trifluopromazine | 1.3 | 15000 |

* Fold disparity between observed compound sensitivity via MTT (Panel A) and apparent receptor affinity (PDSP).

**FIGURE 1** $D_2R$ antagonists induce cytotoxicity in glioma cell lines. A, 50% growth inhibitory concentrations ($IC_{50}$s) for a panel of $D_2R$ antagonists of varying chemotype were determined at 48 hours in a panel of glioma cell lines. $IC_{50}$s were determined via curve-fitting of MTT concentration response curves generated from 100 nmol/L to 100 μmol/L. These values are presented as a heat map for ease of comparison. Best fit curves were fitted with GraphPad Prism. B, Cell lines showed sensitivity to these compounds at micromolar concentrations, whereas they typically have nanomolar affinities ($K_i$) for the $D_2R$. A table of $hD_2R$ affinities for tested ligands[9,10] is presented, along with the fold disparity between observed $IC_{50}$ concentrations (from A) and $K_i$. Cytotoxic activity typically is observed only at concentrations of compounds which would be expected to be well beyond fully saturating at the $D_2R$ and show high nonselective binding to many other receptors (A and B). C, 4 hours MTT concentration response curve for thioridazine in U87MG, showing cytotoxicity within 4 hours. D, Cell counts for U87MG cells under treatment with 10 μmol/L $D_2R$ antagonists. Cells were plated at 20 000 cells/well in 24 well plates and incubated for 24 hours before treatment (treatment at $t = 0$ hour). Data shown are representative of three independent experiments. ****$P < .0001$ via two-way ANOVA with Dunnett's post hoc

receptor antagonists are known to cross the blood-brain barrier and build up within the brain, leading to higher concentrations than are found within the bloodstream. The dopamine D2 receptor family and the associated G protein have also been identified as a potential contributor to GBM in large scale, unbiased screens.[5,6] Here, we investigate the presence and function of $D_2R$ in a broad range of established CNS cancer cell lines and the cytotoxic properties of a number of $D_2R$ antagonists, finding that $D_2R$ is present and functional in many of the cell lines studied, but that $D_2R$ antagonist cytotoxicity is dependent on calcium signaling in a manner that is independent of known $D_2R$ signaling effects.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture and reagents

U87MG, U251MG, A-172, Hs683, and LN-18 cell lines were obtained from ATCC (Manassas, VA, USA). U373MG Uppsala cells were from Sigma Aldrich (St. Louis, MO, USA), and SF-295 cells were obtained from Addex Bio (San Diego, CA, USA). The U87MG, U251MG, and U373MG cells were grown in MEM (Gibco), whereas A-172, Hs683, and LN-18 cells were grown in DMEM, and SF-295 cells maintained in RPMI 1640 (Gibco, Waltham, MA, USA). All cell lines were maintained in media with 10% fetal bovine serum added (Hyclone, ThermoFisher Scientific, Waltham, MA, USA) at 37°C and 5% $CO_2$. All experiments were done with clones used within 20 passages of receipt.

$D_2R$ antagonists haloperidol (McNeill Pharmaceuticals, now part of Johnson and Johnson), pimozide (Sigma Aldrich), thioridazine (Sandoz Pharmaceuticals, now part of Novartis), chlorpromazine (Sigma Aldrich), triflupromazine (SmithKlineFrench, now part of GlaxoSmithKline), clozapine (Sandoz Pharmaceuticals), and remoxipride hydrochloride (Astra AB, now part of AstraZeneca) were dissolved in DMSO to a final concentration of 10 mmol/L, then serially diluted to final concentrations in media and DMSO to control for vehicle effects. $D_2R$ agonists ropinirole and sumanirole (Sigma Aldrich) were kept at 100 mmol/L in DMSO. Thapsigargin (Sigma Aldrich), fendiline hydrochloride (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and forskolin (Tocris Bioscience, Bristol, UK) were maintained at 10 mmol/L. All drug stocks were stored at −20°C except 10 mmol/L ionomycin (Sigma Aldrich), which was stored at −80°C.

### 2.2 | Proliferative and metabolic assays

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were carried out as previously described. Briefly, 2000 cells were plated in each well of a tissue culture treated 96 well plate. The next day, treatments were mixed as described in fresh media via serial dilution and applied to the cells for the given period of time. MTT reagent was added to each well four hours before given experimental time (ie, at 44 hours for a 48 hours MTT assay). Unless otherwise noted, all MTT assays were carried out for 48 hours. Results are presented as normalized values to vehicle treated wells to better enable comparison between cell lines.

BrdU (bromodeoxyuridine) incorporation assays were carried out via ELISA (Millipore Sigma, kit 2750, Burlington, MA) according to the manufacturer's instructions. Cells were plated at 4000 cells/well overnight before treatment and BrdU addition. BrdU experiments were incubated for 24 hours before harvest in lysis buffer, quantification, and analysis. Results are normalized to vehicle control responses to enable comparison between cell lines.

For cell counts, 20 000 cells per well were plated in tissue culture treated 24 well plates. The next day, cells were treated as described. At given time points, cells were rinsed with PBS, detached from the plate with trypsin, and diluted in complete media before manual counting with a hemocytometer. For each independent experiment, three biological replicates were counted in duplicate per treatment group.

### 2.3 | Overexpression and knockdown

For most experiments, stable cell lines expressing *DRD2* shRNA or stably overexpressing *DRD2* were used. To generate these lines, commercially available *DRD2* GIPZ-shRNA viral particles (Dharmacon, Lafayette, CO, USA) or myc-DDK tagged *DRD2* lentiORF particles (Origene Technologies, Rockville, MD, USA) were used according to the manufacturer's recommendations, using 5 μg of particles (titer > $10^7$ TU/mL) per 10 cm dish, followed by puromycin selection. Transient overexpression with GFP-*DRD2* [Addgene #24099[11]] for Figure S3 was achieved with Lipofectamine 3000 according to the manufacturer's recommendations, using the higher recommended concentrations of lipofectamine. Cells were incubated with either 1 μg (for six well plates used in western blotting) or 200 ng (for 96-well plate-based assays, such as MTT assays) DNA for 4 hours before a media change was carried out. At 24 hours after initial transfection, cells were used for their respective assays.

### 2.4 | Cyclic AMP quantification

Cyclic AMP was quantified by ELISA following the supplier's instructions (EMD Millipore, Burlington, MA). Briefly, cells were plated at 10 000 cells/well in 24-well plates overnight, then incubated for 2 hours with 10 μmol/L forskolin and treated with varying concentrations of $D_2R$ agonists before being harvested in 250 μL 0.1 N hydrochloric acid. Data were quantified by a full-range standard curve.

### 2.5 | Flow cytometry

Annexin V/7-AAD flow cytometry was carried out at given time points according to the manufacturer's protocol with annexin

V-PE, 7-amino-actinomycin D, and annexin binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). Samples were tested on the PE and Cy-5 channels on a FACSCanto 10 flow cytometer (BD Bioscience, San Jose, CA, USA) in the Penn State Hershey Flow Cytometry Core within an hour of staining and analyzed with FlowJo v10.

JC-1 staining was carried out with a MitoProbe JC-1 Assay Kit (Thermo Fisher Scientific). Cells were harvested, resuspended to $10^6$ cells/mL in warm PBS, and treated for 15 minutes before a 30 minutes incubation with JC-1 at a final concentration of 2 µmol/L. Cells were then washed with PBS and resuspended in 500 µL of PBS before reading fluorescence in the Cy5 and FITC channels on a FACSCanto 10 flow cytometer (BD Bioscience) in the Penn State Hershey Flow Cytometry Core and analyzed with FlowJo v10.

Cytochrome C flow cytometry was carried out as previously reported.[12] Briefly, cells were plated in 10 cm dishes and incubated overnight before treatment as noted. Cells were harvested with 0.05% trypsin and washed with phosphate buffer saline (PBS) then permeabilized with digitonin permeabilization buffer (100 mmol/L KCl, 50 µg/mL digitonin) for 5 minutes on ice. Cells were then fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature then washed three times with PBS. After blocking for 1 hour in 3% BSA + 0.05% saponin in PBS, cells were incubated overnight at 4°C in a 1:50 dilution of rabbit anti-cytochrome C antibody (136F3, 1:50 dilution, Cell Signaling Technology, Danvers, MA, USA). After three PBS washes, cells were incubated with 1:500 AlexaFluor 488 conjugated F(ab')2 goat anti-rabbit IgG (H + L) secondary antibody (ThermoFisher Scientific) for 30 minutes at room temperature then rinsed two more times before reading fluorescence on the FITC channel of a FACSCanto 10 flow cytometer (BD Bioscience). For all flow cytometric experiments, data represents 2-3 experiments carried out in triplicate, with at least 5000 events per replicate (minimum of 15 000 cells per experiment).

## 2.6 | Immunoblotting

Western blot analysis was carried out as described previously using the ScanLater (Molecular Devices, San Jose, CA, USA) system.[13] Membranes were incubated with primary antibodies for $D_2R$ (1:1000 dilution, Millipore Sigma, Burlington, MA, USA), and vinculin (1:1000 dilution, Cell Signaling Technology) overnight at 4°C after blocking. Eu-conjugated secondary antibodies (Molecular Devices) were applied for one hour at room temperature before rinsing, drying, and reading membranes on a SpectraMax i3x (Molecular Devices). Densitometry was quantified and normalized to the loading control, vinculin, with ImageStudio Lite (LI-COR, Lincoln, NE, USA). The dopamine receptor antibody used was selected due to previous validation published by another research group CITE, though the authors accede that the most accurate way to measure D2R surface expression would be through radioligand binding assay due to the poor quality of many dopamine receptor antibodies.

## 2.7 | qRT-PCR

Quantitative PCR was carried out in 384-well plates with commercially available TaqMan probes according to manufacturer's instructions using an ABI QuantStudio 12K Flex system located in the Genomics Core at the Penn State Hershey.

## 2.8 | Calcium imaging

To measure single cell cytosolic $Ca^{2+}$, U87MG cells were cultured on 30-mm glass coverslips for 24 hours in complete media.[14] Coverslips were then mounted into nonstick PTFE imaging chambers and incubated for 30 minutes at 37°C in complete media containing 2 µmol/L of Fura-2 AM. Using HEPES-buffered saline solution (HBSS; 140 mmol/L NaCl, 1.13 mmol/L $MgCl_2$, 4.7 mmol/L KCl, ±2 mmol/L $CaCl_2$, 10 mmol/L D-glucose, and 10 mmol/L HEPES; pH 7.4), cells were washed four times, and kept in this solution for 10 minutes at room temperature prior to imaging. A digital fluorescence imaging system (InCytIm2; Intracellular Imaging, Cincinnati, OH, USA) was used to measure fluorescence from single cells. The dye was excited alternately at 340 and 380 nm and fluorescence recorded at 510 nm. The emission ratio of 340/380 nm of each pixel was used to represent the $Ca^{2+}$ signal. Compounds were suspended to listed concentrations in HBSS. $Ca^{2+}$ traces represent the averages from at least 5-10 cells per coverslip and 2-4 independent experiments.

Similarly, to measure maximal $Ca^{2+}$ responses to thioridazine, FLIPR (fluorescence imaging plate reader) assays were used. Cells were cultured in 96-well plates for 24 hours in complete media. 96-well plates were then incubated for 30 minutes at 37°C in complete media containing 2 µmol/L of Fura-2 AM. Cells were then washed four times with HEPES buffered saline solution, and kept in this solution for 10 minutes at room temperature. Plates were loaded into FlexStation 3 and alternately excited at 340 and 380 nm and emitted fluorescence was recorded at 510 nm. Maximal $Ca^{2+}$ was calculated at peak fluorescent signals and normalized to fluorescence at time zero.

For measurements of mitochondrial and ER $Ca^{2+}$, U87MG cells were cotransfected with 1 µg of R-CEPIA1er and G-CEPIA2mt and seeded on 30-mm glass coverslips for 24 hours in complete media.[15] Coverslips were then mounted to Teflon chambers and submerged with HBSS. To measure fluorescence from R-CEPIA1er and G-CEPIA2mt, a TCS SP8 (Leica, Wetzlar, Germany) equipped with a 63× objective captured images at the 552 nm/560-800 nm and 488 nm/500-550 nm excitation/emission wavelengths, respectively. Leica X software (Wetzlar, Germany) was used to process and analyze images. $Ca^{2+}$ signal was represented by normalizing each measurement by fluorescence recorded at time zero.

## 2.9 | Quantification and statistical analysis

Data are presented as mean ± SEM. All experiments had 3 independent replications unless otherwise noted, and in each experiment
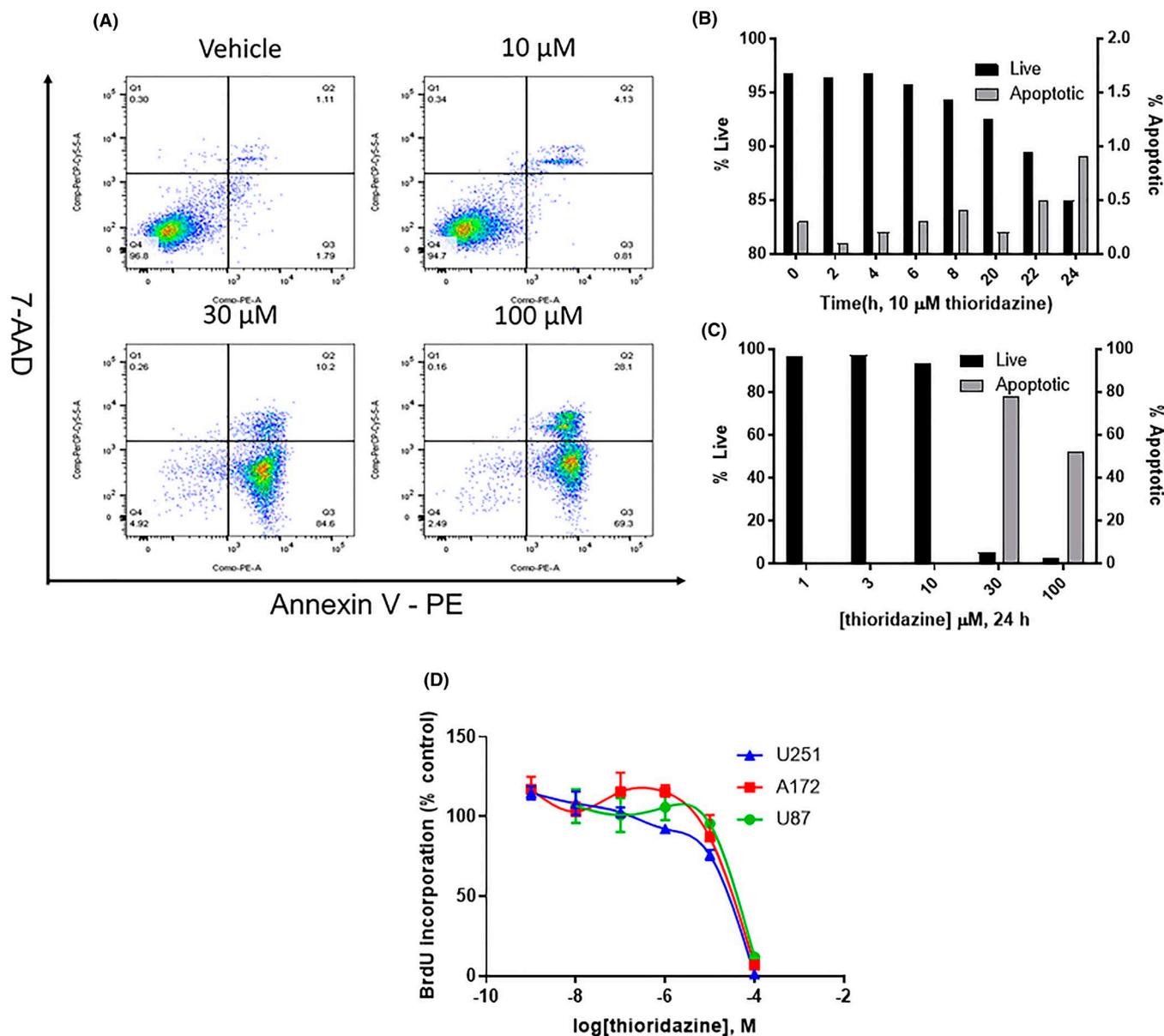
**FIGURE 2** $D_2R$ antagonists induce apoptosis in glioma cell lines. A, Representative plots are presented for annexin V/7-AAD flow cytometry, measuring apoptotic induction in U251MG cells under treatment with 0, 10, 30, or 100 µmol/L thioridazine for 24 hours. Induction of apoptosis in U87MG cells via treatment with 10 µmol/L thioridazine is time B, and concentration (C) dependent. Flow cytometric experiments were carried out within 1 hour of harvest on a FACSCanto10 flow cytometer, using the PE and Cy-5 fluorescence channels, gating for whole single cells. Data workup was performed with FlowJo v10. D, 24 hours BrdU incorporation assays were carried out using ELISA methods in U87MG, U251MG, and A-172 cell lines according to standard protocols. Proliferation (D) is not significantly affected by $D_2R$ antagonist treatment until concentrations at which widespread cell death is seen by flow cytometry (A-C)

triplicate samples or more were used. Statistical analysis was carried out with one- or two-way ANOVA followed by multiple comparisons with Dunnett's post hoc test to compare against controls, or Sidak's post hoc test to compare within treatment groups.

## 2.10 | Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal from the IUPHAR/BPS Guide to

PHARMACOLOGY,[16] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/2020.[17]

## 3 | RESULTS

### 3.1 | $D_2R$ antagonists induce cytotoxicity in glioblastoma cell lines

First, we investigated whether the anticancer activity of $D_2R$ antagonists was restricted to one chemotype or caused by $D_2R$-targeted

ligands from several chemical families. We studied chemotypes that included phenothiazines (thioridazine, chlorpromazine, triflupromazine, metopimazine), phenylbutylpiperidines (haloperidol), diphenylbutyl-piperidines (pimozide), substituted benzamides (remoxipride), and tricyclic benzodiazepines (clozapine). Using a 48 hours MTT assay screening, most of the compounds tested decreased MTT activity with IC50 values ranging from 2 to 20 μmol/L (Figure 1A). Except for thioridazine, the $IC_{50}$ values were very similar for each compound in the six cell lines tested, and even for thioridazine the EC50 values were within a ten-fold range. Of note, for each of the six compounds that caused cytotoxicity, the $IC_{50}$ values we calculated were 10-25 000 times higher than the reported $K_D$'s at the $D_2R$ (Figure 1B). The exception, remoxipride, had no activity in this assay at any tested concentration (Figure 1B). In addition, except for thioridazine (Figure 1C), the active compounds all had steep dose-response curves (Figure S1A) that are atypical for GPCR-mediated effects.

We then exposed cells for 24 hours, and used annexin V/7-AAD flow cytometry to determine the effects of these compounds on apoptosis. After treatment with thioridazine, pimozide, or haloperidol, cell lines A172, U251, and U87MG all showed signs of apoptosis and necrosis that occurred in a time- and concentration dependent manner (Figure 2A-C, Figure S1B and C). Cell proliferation, as measured by BrdU (bromodeoxyuridine) incorporation assays, was not negatively impacted until widespread, early-onset cell death was observed (Figure 2D). Interestingly, remoxipride had no effect on cell metabolic activity, even at concentrations much higher than toxic levels (Figure 3). This is notable, as remoxipride is more $D_2R$ selective than the other tested compounds. These latter data are inconsistent with the hypothesis that the $D_2R$ is mediating the cytotoxicity of these $D_2R$ antagonists.

## 3.2 | Dopamine $D_2$ receptors are expressed and functional in glioblastoma cell lines

If the hypothesis that the $D_2R$ mediates anticancer effects were true, it would predict that different expression levels of this protein might affect compound response between cell lines, potentially explaining
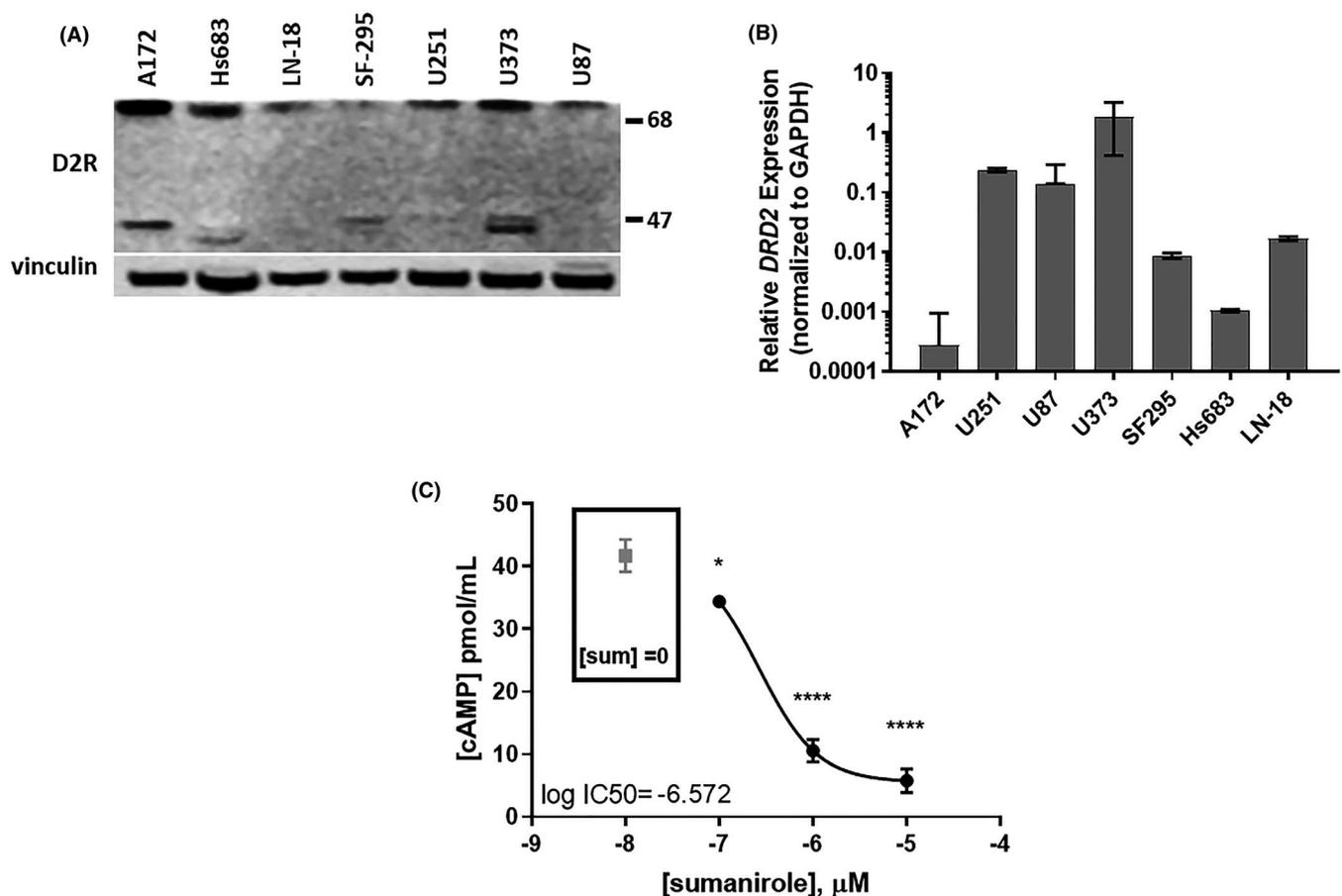


**FIGURE 3** Dopamine $D_2$ receptor is present and signals in glioma cell lines. A, Western blots indicate the presence of $D_2R$ in both mature, glycosylated states (~80 kDa) and immature, lower molecular weight species (~47 kDa) in untreated glioma cell lines (quantified in Figure S1). B, qrt-PCR for *DRD2* and *GAPDH* transcripts were carried out in untreated glioma cell lines using standard TaqMan probes. Standard deviations for GAPDH expression were less than 0.085. C, 100-1000 nmol/L $D_2R$ agonist sumanirole for 2 hours reduces forskolin-stimulated cytoplasmic cAMP levels in U87MG, with a IC50 of 250 nmol/L, compared to previously reported EC50 values of 17-75 nmol/L for cell based assays in $D_2R$ expressing CHO cells.[31] Data are representative of 2-3 independent experiments in triplicate. *P < .05; ****P < .001 via two-way ANOVA with Dunnett's post hoc
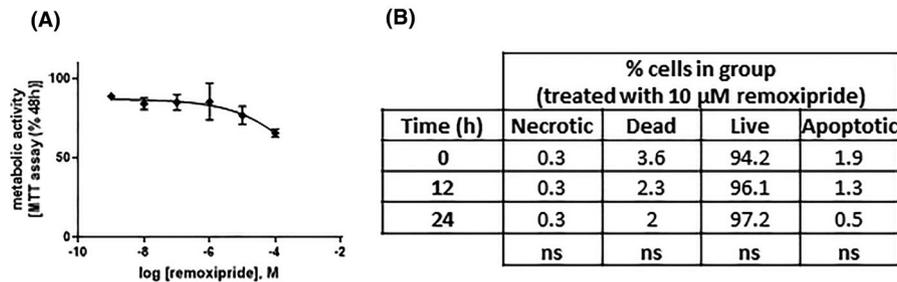
**(A)**



**(B)**

| Time (h) | % cells in group (treated with 10 µM remoxipride) | | | |
|---|---|---|---|---|
| | Necrotic | Dead | Live | Apoptotic |
| 0 | 0.3 | 3.6 | 94.2 | 1.9 |
| 12 | 0.3 | 2.3 | 96.1 | 1.3 |
| 24 | 0.3 | 2 | 97.2 | 0.5 |
| | ns | ns | ns | ns |

**FIGURE 4** Selective $D_2R$ antagonist, remoxipride, does not induce cytotoxicity in glioma cell lines. A, Cells were treated with 1 nmol/L to 100 µmol/L remoxipride for 48 hours to generate an MTT concentration response curve. No $IC_{50}$ could be calculated from generated data due to lack of effect at tested concentrations, but it would be greater than 100 µmol/L. B, At 12-24 hours, 10 µmol/L remoxipride does not significantly affect the induction of apoptosis by thioridazine as seen by annexin V/7-AAD flow cytometry. Data are representative of three or more independent experiments in triplicate for MTT and BrdU experiments, while flow cytometry experiments are representative of three independent experiments of 5000-10 000 cells. Two-way ANOVA with Sidak's post hoc was used to analyze significance. ns, not significant via two-way ANOVA with Sidak's post hoc
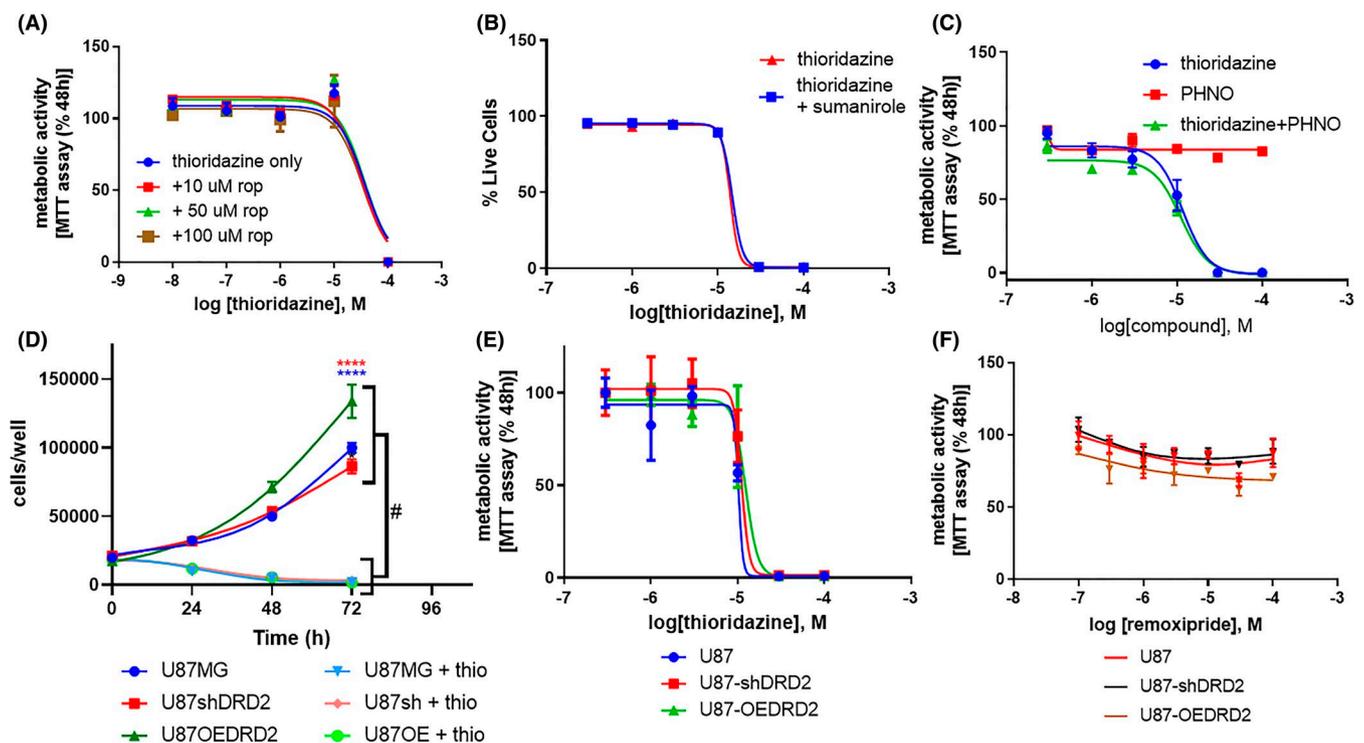


**FIGURE 5** $D_2R$ is not involved in cytotoxic responses to $D_2R$ antagonists. A, 48 hours MTT concentration response curves were generated from 10 nmol/L to 100 µmol/L for thioridazine in U87MG cells in the presence of 0, 10, 50, or 100 µmol/L ropinirole, a $D_2R$ agonist. The presence of ropinirole did not significantly alter thioridazine sensitivity. B, Cotreatment with 30 µmol/L sumanirole, a $D_2R$ agonist, did not significantly reduce the induction of apoptosis in U87MG cells treated with 0.3-100 µmol/L thioridazine for 24 hours and analyzed via annexin V/&-AAD flow cytometry. C, Cotreatment with 30 µmol/L PHNO, a $D_2R$ agonist, did not significantly alter sensitivity of U87MG cells to 0.3-100 µmol/L pimozide according to 48 hours MTT concentration response curves. D, Knockdown of *DRD2* by ~50% (Figure S3D) in U87MG cells via stable lentiviral transduction reduces cell proliferation slightly, but does not significantly affect response to 10 µmol/L thioridazine as measured by cell counts. E, 48 hours MTT concentration response curves are presented for U87MG-DRDOE and U87MG-DRD2KD cells under treatment with 0.3-100 µmol/L thioridazine. No significant differences in response were noted from parental cells. F, 48 hours concentration response curves for U87MG, U87MG-DRDOE and U87MG-DRD2KD cells treated with 0.1-100 µmol/L remoxipride. No significant sensitivity is noted in any cell line used. Data are representative of two independent experiments carried out in triplicate and is analyzed by two-way ANOVA with Sidak's post hoc. ****$P < .0001$ via two-way ANOVA with Sidak's post hoc vs control for series of matched color.; #$P < .0001$ for matched treatment pairs via two-way ANOVA with Sidak's post hoc

cell line differences in $D_2R$ antagonist cytotoxic responses. We thus studied the presence and signaling of $D_2R$ in a panel of glioma cell lines, including U87MG, U251MG, U373MG-Uppsala, Hs683, SF-295,

A172, and LN-18. Although the presence of the $D_2R$ was seen at both the protein and message level in all of the cell lines, expression levels varied markedly (Figure 3A and B). Some cell lines had more mature

glycosylated $D_2R$ (A172), whereas others had lower overall levels of $D_2R$ or predominantly unglycosylated protein (SF-295) that may not achieve proper cellular localization for signaling, although verification of the identity by mass spectrometry was not done.[18] Functionally, the $D_2R$ agonists sumanirole, PHNO, or ropinirole significantly reduced intracellular cyclic AMP levels in forskolin-stimulated GBM cells ($P < .01$, one-way ANOVA with Sidak's post hoc), suggesting that $D_2R$ were present and capable of signaling (Figure 3C, Figure S2A-C). U87MG cells appear less sensitive than A172 and U251 cells to ropinirole and PHNO, an effect which may be due to varying levels of D2R or other dopamine receptors at which these compounds may have activity. Thus, $D_2R$ are present albeit at low levels in these GBM cell lines and show expected functional activity to $D_2R$ agonists.

### 3.3 | Overexpression and knockdown of $D_2R$ did not alter compound response

Next, we used $D_2R$ agonist competition to determine if this would attenuate the actions of the antipsychotic drugs presumed to act as $D_2R$ antagonists. The addition of $D_2R$-saturating but non-cytotoxic concentrations of ropinirole, sumanirole, or PHNO had no effect on the cytotoxic responses of cells to antagonists as assessed by the MTT assay (Figure 5A-C, Figure S3A-C). To gain insight into possible off-target activity, the *DRD2* was then either overexpressed or knocked down in U87MG cells (Figure 5D, Figure S3D). Knockdown slightly reduced cell proliferation rates as measured by cell counts (Figure 3D, Figure S3D-F, $P < .01$ via two-way ANOVA with Sidak's post hoc) as is known,[5] but compound response was not affected by either the overexpression or knockdown (Figure 5D-F, Figure S3D-F). As before, remoxipride had no effect on cell proliferation regardless of co-treatment or genetic alterations (Figures 4 and 5F). Similar effects were seen with transient transfection of $D_2R$ in U87MG (Figure S3G). $D_2R$ knockdown in U251MG or A172 cells also did not affect compound responses (Figure S3B and C, E and F, H and I). Thus, neither pharmacological competition, nor genetic manipulation of the $D_2R$, was able to rescue cells from the cytotoxicity induced by the antagonists, further supporting the hypothesis that the actions of these compounds were mediated by a mechanism other than blockage of the $D_2R$.

### 3.4 | Calcium flux is altered in $D_2R$ antagonist-treated glioblastoma cell lines

These data, coupled with near quantal dose-response curves atypical of $D_2R$, suggest that the $D_2R$ antagonists are working via another mechanism(s). As many of these compounds are known to affect ion flux within cells, we focused on the potential role of calcium signaling that is known to be altered in cancer cells.[19,20] In particular, the peripheral side effects of thioridazine have been suggested to involve calcium channel antagonism.[21] Co-treatment with 3 µmol/L fendiline, a nonselective calcium channel blocker and calmodulin antagonist, sensitized U87MG and A172 cells to the $D_2R$ antagonist thioridazine

(Figure 6A, Figure S3B, $P < .05$ via one-way ANOVA with Sidak's post hoc). Thioridazine has been repeatedly identified as a potential anti-cancer therapeutic in recent years, and is one of the most well-characterized antipsychotics in the context of cancer.[7,22,23] This suggested that broad disruption of the typically highly regulated calcium signaling pathways may be involved in the anticancer mechanism of action for such compounds, as has been previously suggested.[4,24,25] Indeed, treatment with antipsychotics, especially the phenothiazines, induced a rapid and sustained calcium response far beyond that induced by 2 µmol/L of the SERCA blocker thapsigargin, which prevents uptake of calcium by the ER (Figure S4A), either in the presence, or absence, of extracellular 2 mmol/L calcium (Figure 6B-D). These responses suggest that phenothiazines may enhance cytosolic calcium by altering calcium transport across both the endoplasmic reticulum membrane (the major $Ca^{++}$ storage site within the cell) and the plasma membrane, as shown by the increase in both the presence and absence of extracellular $Ca^{++}$. Remoxipride, however, had no such effect (Figure 4E), and genetic overexpression or knockdown of $D_2R$ did not significantly alter calcium responses (Figure 4F, Figure S4B), suggesting that $D_2R$ is not required for these alterations.

### 3.5 | Calcium signaling is involved in the anticancer mechanism of action for $D_2R$ antagonists

Although these $D_2R$ antagonists altered the calcium signal in glioma cell lines by a non-$D_2R$ mechanism, it is unclear if the calcium signal is an epiphenomenon unrelated to the cell toxicity. Thus, BAPTA-AM was used to chelate cytosolic calcium. Adequate BAPTA-AM preloading was determined via FLIPR concentration response (Figure 7A). At a concentration that blocks the cytosolic calcium increase from the well-studied positive control, thapsigargin, by ~50%-70%, BAPTA-AM preloading significantly reduced cytotoxic responses in U87MG assessed by both MTT and cell count (Figure 7B and C, $P < .05$ via two-way ANOVA with Sidak's posthoc). To study the anticancer mechanism of action for these compounds further, mitochondria- and ER-targeted genetically-encoded calcium dyes CEPIA-mt2 and R-CEPIA-ER (CEPIA; calcium-measuring organelle-entrapped protein indicators) were transfected into cells, and used to determine calcium levels in the mitochondria and ER of U87MG cells.[15] This method allows for simultaneous imaging of calcium levels in the endoplasmic reticulum (ER) and the mitochondria (Figure 7D). Upon treatment with 10 µmol/L thioridazine, calcium signals from the ER were reduced, indicating the emptying of ER stores (Figure 7E). The mitochondrial calcium signal, however, increased over this time, suggesting that the calcium released from the ER was taken up by the mitochondria. This is similar to the action of 2 µmol/L thapsigargin (Figure S4C) that selectively inhibits SERCA pumps within the ER membrane, and leads to calcium release into the cytoplasm. This cytosolic calcium then is buffered by mitochondria, and was sufficient to disrupt the mitochondrial membrane potential within 45 minutes, as determined by JC-1 staining (Figure 7F). Cytochrome c was consequently released within 4 hours (Figure 7G). Similar results were seen in cell lines A172 and
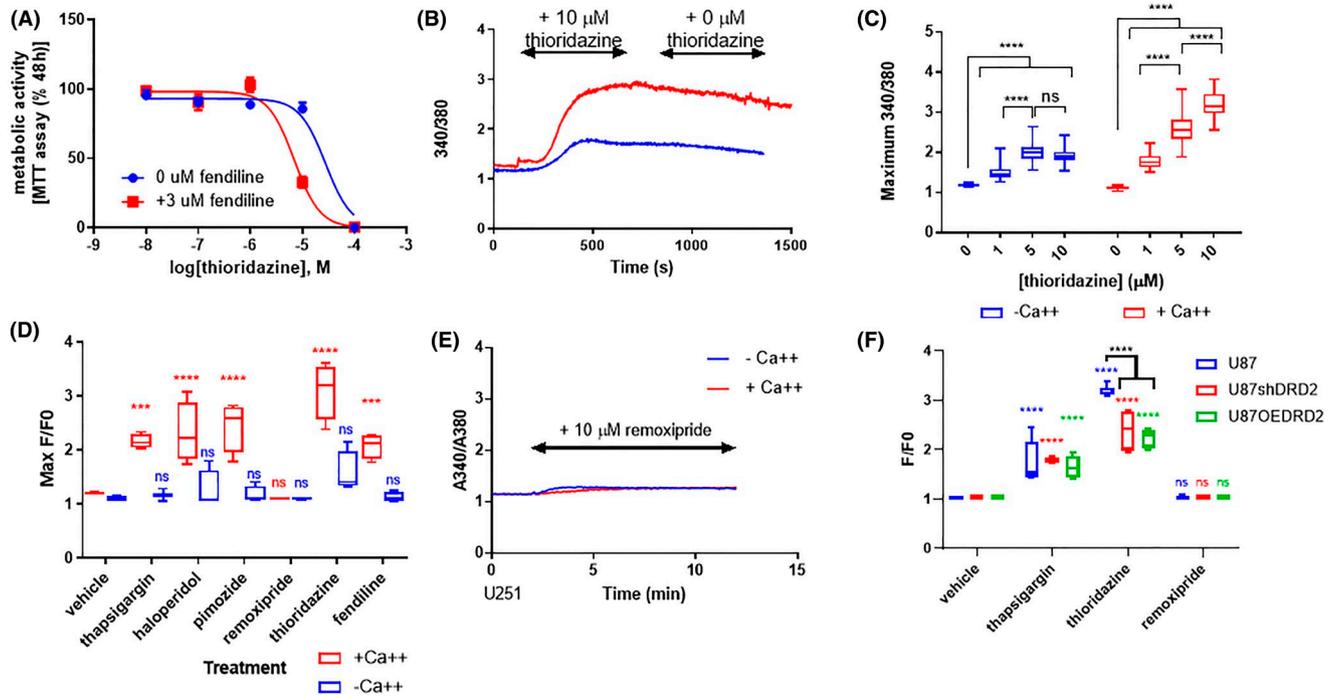
**FIGURE 6** $D_2R$ antagonists, other than remoxipride, alter calcium signaling in glioma cell lines. A, U87MG cells were treated with 0.1-100 μmol/L thioridazine for 48 hours in the presence or absence of 3 μmol/L fendiline, a nonspecific calcium channel blocker and calmodulin inhibitor. MTT concentration response curves suggest a significant sensitization to thioridazine in cells co-treated with fendiline. Values shown are normalized to vehicle treated cells and represent three independent experiments. B, 10 μmol/L thioridazine induces a strong and sustained increase in cytosolic calcium release as measured by single cell imaging of U87MG cells preloaded with 2 μmol/L Fura-2 AM for 30 minutes. This increase is due to both ER calcium release (as shown by the increase in the absence of extracellular calcium, in red) and calcium influx from the extracellular space (2 mmol/L $CaCl_2$, in blue). Unlike the positive control, 2 μmol/L thapsigargin (Figure S4A), the cell is unable to clear this signal for a significant amount of time. Traces are presented as the ratio of fluorescence at 340 and 380 nm. C, Thioridazine increases cytosolic calcium signal in a concentration dependent manner in the presence of 0 or 2 mmol/L $CaCl_2$ as measured by Fura-2 AM fluorescence (shown as maximum ratio of fluorescence at 340 and 380 nm) of U87MG cells preloaded with Fura-2 AM for 30 minutes as measured by FLIPR assay. D, Maximal calcium responses of selected compounds as measured by Fura-2 AM FLIPR assay are presented. All compounds induced increases in cytosolic $[Ca^{++}]$ in the presence of 2 mmol/L $CaCl_2$ except for remoxipride. E, Remoxipride does not affect cytoplasmic calcium levels as measured by single cell imaging with Fura-2 AM as in (B). F, Maximal calcium responses are not significantly altered by overexpression or knockdown of *DRD2* in FLIPR imaging of Fura-2 AM as in C. A is representative of three experiments in triplicate, while single cell imaging experiments are representative of three independent experiments with at least two cover slips and 40 cells. FLIPR assays are representative of at least two independent experiments carried out in quadruplicate. $*P < .05$; $**P < .01$; $***P < .005$; $****P < .0001$ via two-way ANOVA with Sidak's post hoc

U251MG (Figure S4D-I). These results provide strong evidence for a calcium-mediated mechanism for cytotoxicity induced by thioridazine.

## 4 | DISCUSSION

### 4.1 | $D_2R$ antagonists do not cause cytotoxicity in glioblastoma cell lines via $D_2$ receptor mechanisms

This study tested the hypothesis that antagonism of the $D_2$ dopamine receptor mediates the anticancer cytotoxicity that has been reported for many repurposed antipsychotic drugs. We tested more than a half-dozen approved or experimental antipsychotics representing four distinct chemotypes, and found that all of these drugs, with the exception of remoxipride, were indeed cytotoxic to each of the six cell lines tested. The fact that remoxipride did not show similar cytotoxicity at first appears to be an anomaly, until we

compared the known affinities of these compounds with their cytotoxic $EC_{50}$. For each drug that had cytotoxic efficacy, the EC50 was 2-4 orders-of-magnitude greater than the $K_D$ for the $D_2R$. In addition, the dose-response curves for these molecules were very steep, inconsistent with the typical slope that is seen in the interaction of antagonists with the $D_2R$. These findings are inconsistent with the hypothesis that $D_2R$ antagonism is a novel approach to treatment of brain gliomas.

One of the facts that might influence the potency of these compounds if actually working via $D_2$ receptors is the level of $D_2R$ expression in the various lines. In the seven lines we studied, evidence for expression of the $D_2R$ was seen at both the protein and message level in all of the cell lines. Expression levels varied markedly and were unrelated to the effects of these agents. The $D_2Rs$, even when present at very low expression levels in these GBM cell lines, showed typical responses to $D_2R$ agonist induced canonical signaling. Because these $D_2R$ appeared functional, then if the antipsychotics were acting via
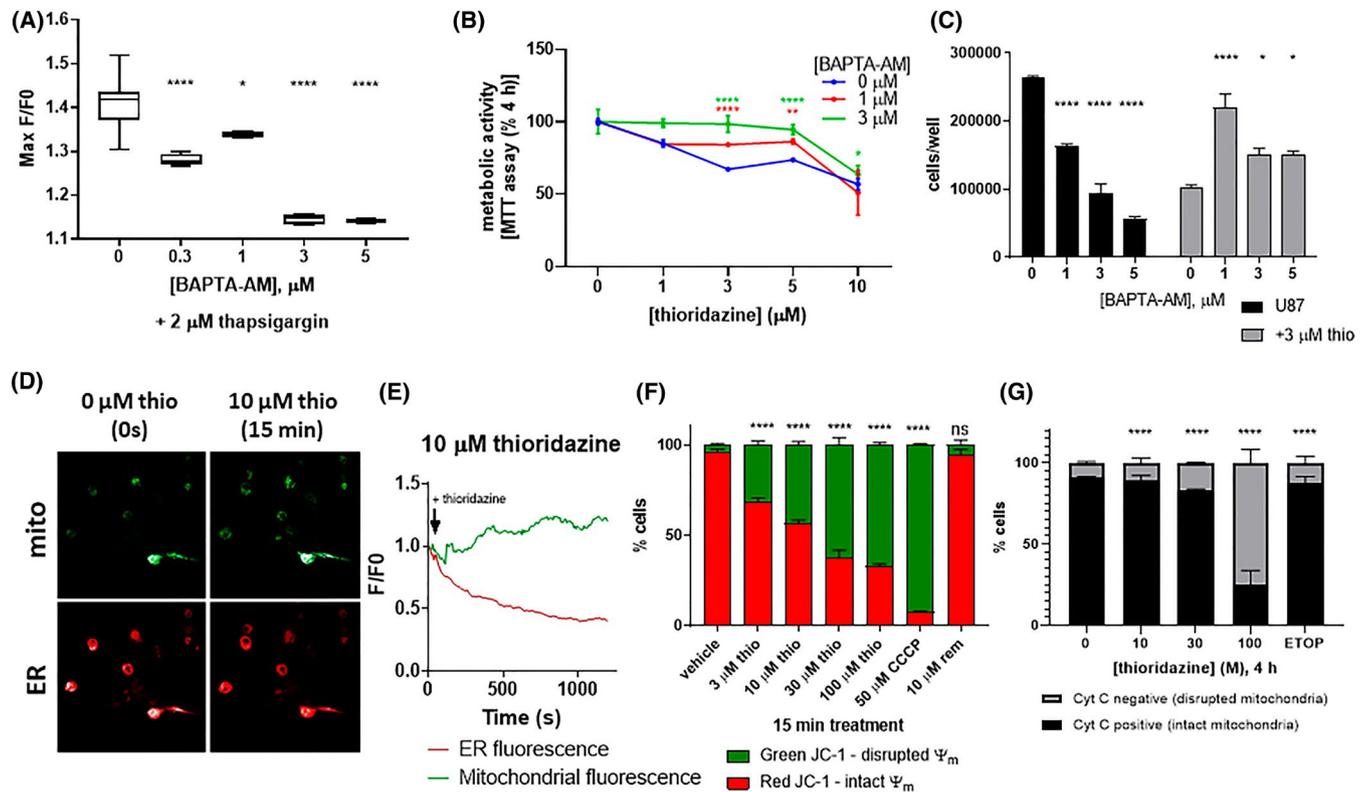
**FIGURE 7** Calcium signaling is involved in thioridazine-induced cytotoxicity. A, Pretreatment of 4000 U87MG cells for 30 minutes with 0-5 μmol/L BAPTA-AM concentration-dependently reduced cytoplasmic calcium signal from 2 μmol/L thapsigargin exposure in U87MG cells via FLIPR assay with Fura-2 AM preloaded cells. B, 4 hours MTT response for 0-10 μmol/L thioridazine under cotreatment with 0-3 μmol/L BAPTA-AM. C, Pretreatment with 1-5 μmol/L BAPTA-AM for 20 minutes reduces the induction of apoptosis by 10 μmol/L thioridazine in U87MG cells via 24 hours cell counts. D, Representative images from CEPIAmt2 and CEPIAer stained U87MG cells treated with 10 μmol/L thioridazine for 0-15 minutes. E, Quantification of CEPIA staining in U87MG under treatment with 10 μmol/L thioridazine. Results are presented as the ratio of fluorescence over initial fluorescence (F/$F_0$). Similar results were seen with 2 μmol/L thapsigargin (Figure S4C). F, JC-1 staining for mitochondrial potential indicates a concentration dependent loss of mitochondrial potential within 15 minutes under treatment with 0-100 μmol/L thioridazine. CCCP, a protonophore, serves as a positive control. 10 μmol/L remoxipride had no effect on mitochondrial membrane potential. G, Release of cytochrome c is induced by treatment with 10-100 μmol/L thioridazine for 4 hours, as measured by flow cytometry with staining for cytochrome c, to equivalent or greater levels than the positive control, 30 μmol/L etoposide. Experiments were analyzed via two-way ANOVA with Dunnett's post hoc. *$P < .05$; **$P < .01$; ***$P < .005$; ****$P < .0001$ via two-way ANOVA with Sidak's post hoc

the D2 receptor, their actions should have been affected by addition of $D_2R$ agonists (either moving the concentration response curve left or right). The agonists, at near-saturating, but non-cytotoxic, concentrations, however, had no effect on the cytotoxicity of the antagonists. This was additional evidence against the hypothesis that the cytotoxicity was a result of $D_2R$-mediated mechanisms, and suggested that another target(s) was responsible. Consistent with this, molecular manipulation of $D_2R$ expression (either over-expression or knockdown) affected the cytotoxicity induced by the antagonists, further evidence for an off-target, non-$D_2R$ mechanism.

## 4.2 | Calcium signaling: an alternate mechanisms for the actions of $D_2R$ antagonists

Calcium channel antagonism had been proposed to play a role in the peripheral side effects of thioridazine,[21] thus providing an alternate hypothesis for the actions of these drugs. Since calcium signaling is

known to be altered in cancer cells,[19,20] we investigated this mechanism. Here, we focused on thioridazine because it often has been suggested to be a potential anticancer therapeutic.[7,22,23] Fendiline, a nonselective calcium channel blocker and calmodulin antagonist, sensitized the U87MG and A172 cells to thioridazine consistent with the notion that calcium signaling may contribute to a potential mechanism of anticancer cytotoxicity.[4,24,25] Indeed, treatment with antipsychotics, especially the phenothiazines, induced a rapid and sustained calcium response by altering cytoplasmic calcium levels. In the case of thioridazine, calcium ion transport across both the endoplasmic reticulum membrane and the plasma membrane were altered as well, yet genetic overexpression or knockdown of $D_2R$ did not significantly alter calcium responses. Given structural similarities, similar side effect profiles, and cell culture behaviors, as observed by ourselves and others, this effect may be common to the phenothiazine class of drugs. Moreover remoxipride, the $D_{2/3}$ antagonist that did not cause cytotoxicity, also did not affect calcium mobilization. It is possible that the effects on calcium signal in glioma cell lines

also might be an epiphenomenon unrelated to the cell toxicity. Our experiments using fluorescent dyes to image calcium levels in the endoplasmic reticulum and the mitochondria simultaneously suggest, at least for thioridazine, that pertubations of calcium are involved in the cytotoxicity we observed.

## 5 | CONCLUSIONS

The weight of the evidence we have gathered is inconsistent with these putative $D_2R$ antagonists causing cytotoxicity in glial cancer cells lines via a $D_2$ dopamine receptor mechanism. Although we did not specifically rule out a role for other dopamine receptors (especially the $D_3$ and $D_4$ for which these compounds have varying affinities), the basic pharmacological principles we have applied here (specifically the concentrations required for cytotoxicity vs the concentrations that might be feasible in clinical use), would equally disqualify the other dopamine receptors as potential mechanisms. Moreover the agonist reversal experiments provide direct evidence against a role of $D_3$ since two of the compounds used (bromocriptine and ropinirole) have significant $D_3$ affinity.

Here, we focused on already-FDA approved compounds which may be quickly repurposed in the event that a safe, efficacious dosage were to be determined for human patients. These compounds have largely not been explored as anti-GBM therapeutics via clinical trial, with the notable exception of chlorpromazine (NCT04224441). Attempts to repurpose existing drugs that have already been approved or shown safe for use in humans is a useful approach. The value of repurposing is lost if one cannot develop clear evidence that the novel use of the repurposed compounds (in this case antipsychotic drugs) either involves the primary known mechanism of these drugs (in this case the $D_2R$), or occurs at concentrations consistent with what might be achieved clinically. More generally, the importance of concentration in translating cellular studies to whole organisms is well known to pharmacologists, but seems often to not receive adequate consideration.[26]

In the current case, we felt it necessary to provide a rigorous test of the hypothesis that $D_2R$-mediated actions of antipsychotic drugs might yield a novel approach to therapy of brain cancers like GBMs. We had proposed the alternate hypothesis that the cytotoxicity of these compounds was not due to actions via $D_2R$,[10] and the current data suggest that this hypothesis survives, as well as offering calcium-signaling as an alternate mechanism. Indeed, recent studies of ONC201, a compound with dopamine receptor antagonist activity currently in clinical trials (NCT02525692), suggest that this compound may also have a mechanism of action related to mitochondrial calcium flux.[27,28] We also note recent reports suggesting that the dopamine $D_1$ receptor also plays a role in the actions of a novel potential chemotherapeutic.[29] We believe that the same pharmacological reasoning we have used here suggests that these proposed $D_1$ mechanisms are not correct.

While it seems clear that $D_2R$ may not be necessary for the cytotoxic effects of these compounds, it is possible that this family of receptors contributes to a cancer phenotype. As we have previously shown, pharmacological activity of $D_2R$ modulators at more selective 100 nmol/L concentrations, as well as genetic modulation of $D_2R$ expression levels, altered spheroid formation, proliferation, and invasion activities in U87MG cells. $D_2R$ agonists promoted cancer-like behaviors and antagonists reduced them, even at these low concentrations.[30] Such findings are consistent with previous published work suggesting that dopamine receptors contribute to mitogenic signaling in GBM.[5]

If our hypothesis is correct that perturbed calcium function is a/the mechanism by which the antipsychotics caused cytotoxicity, there are two areas of investigation that are immediately apparent. The first is the identification of the specific target(s) within the complex network of mechanisms that can affect calcium signaling. The second is whether these targets actually provide a feasible lead for new drugs because of the ubiquitous nature of calcium signaling in all cells. The latter may be an especially challenging concern, which may be somewhat ameliorated by differences in reliance on calcium signals in cancerous and normal cells.[19]

### DISCLOSURE
We report no conflicting interests for this study.

### AUTHOR CONTRIBUTIONS
JSW designed and carried out all experiments and drafted the manuscript. MTJ and MT provided guidance for calcium imaging experiments. JDN, RBM, and RJH provided insight into experimental design, and edited the manuscript.

### DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.