Thioridazine inhibits self-renewal in breast cancer cells via DRD2-dependent STAT3 inhibition, but induces a $G_1$ arrest independent of DRD2

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Thioridazine is an antipsychotic that has been shown to induce cell death and inhibit self-renewal in a broad spectrum of cancer cells. The mechanisms by which these effects are mediated are currently unknown but are presumed to result from the inhibition of dopamine receptor 2 (DRD2). Here we show that the self-renewal of several, but not all, triple-negative breast cancer cell lines is inhibited by thioridazine. The inhibition of self-renewal by thioridazine in these cells is mediated by DRD2 inhibition. Further, we demonstrate that DRD2 promotes self-renewal in these cells via a STAT3– and IL-6–dependent mechanism. We also show that thioridazine induces a $G_1$ arrest and a loss in cell viability in all tested cell lines. However, the reduction in proliferation and cell viability is independent of DRD2 and STAT3. Our results indicate that although there are cell types in which DRD2 inhibition results in inhibition of STAT3 and self-renewal, the dramatic block in cancer cell proliferation across many cell lines caused by thioridazine treatment is independent of DRD2 inhibition.

The family of dopamine receptors consists of five G protein–coupled receptors (GPCRs)2. There are two subfamilies of dopamine receptors, which couple to different G proteins and can have different effects on signaling. The Gαs-coupled D1-like receptors (DRD1 and DRD5) induce cAMP production, whereas the Gαq-coupled D2-like receptors (DRD2, DRD3, and DRD4) inhibit cAMP production (1, 2). DRD2 in particular has been extensively studied because of its disease relevance. Excess or reduced DRD2 activity is thought to be responsible for diseases such as schizophrenia and Parkinson’s disease, and compounds that preferentially inhibit or activate DRD2, respectively, can be used to manage these diseases (2, 3). Although much of the research on dopamine receptors has focused on neurons, functions for dopamine and its receptors outside of the CNS have been reported. Expression of dopamine receptors has been observed in renal cells, where they regulate inflammation and blood pressure (2, 4, 5). Dopamine receptors, including DRD2, have also been identified in immune cells. The effects of dopamine and its receptors have been especially well-studied in T-cells, where they regulate T-cell activation and proliferation (6–8).

Recently, evidence has emerged that DRD2-targeting antipsychotics can block the growth of several cancer types, including leukemia, glioblastoma, and colorectal, lung, and breast cancer cell lines (9–14). Interestingly, the DRD2-targeting antipsychotic thioridazine had been shown as early as 1992 to inhibit breast cancer cell growth (15). Further, high expression of DRD2 mRNA has been observed in glioblastoma and pancreatic cancer (13, 16). DRD2-targeting antipsychotics such as thioridazine and haloperidol have been reported to induce apoptosis and reduce self-renewal in cancer cells (9–13). Despite these discoveries, the molecular mechanisms by which antipsychotics such as thioridazine lead to reduced cancer cell growth and survival are still unclear. Several studies of the effects of thioridazine have been performed using high concentrations (10–20 μM) of drug (9–12). It remains unclear whether certain cell types may be more or less sensitive to treatment with antipsychotics, whether thioridazine can inhibit self-renewal effects independent of cell toxicity, and whether these effects are mediated by inhibition of DRD2 as is generally assumed. However, it is clear that DRD2-targeting antipsychotic compounds have very strong effects on many cell types.

STAT3 is an inducible transcription factor that has been shown to promote proliferation, prevent apoptosis, and affect cellular differentiation (17). STAT3 is one of six STAT family transcription factors that are primarily known for roles in pathogen response and inflammation (18). Not only has STAT3 been shown to promote self-renewal in cancer stem cells and normal embryonic stem cells, but its activation also increases breast cancer proliferation and invasion (19–23). A number of stimuli are capable of activating STAT3, including the widely studied cytokine interleukin-6 (IL-6). IL-6 forms a complex with IL-6 receptor and GP130 and activates the tyrosine kinase activity of GP130. Active GP130 can phosphorylate and activate...
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members of the JAK tyrosine kinase family, which then activate STAT3 by phosphorylating Tyr-705 (24). Importantly, IL-6 is also a target gene of STAT3, and a feed-forward loop can be established that leads to chronically activated STAT3. Studies indicate that chronically activated STAT3/IL-6 promotes more aggressive and drug-resistant breast cancers (25–27). Although receptor tyrosine kinase signaling is a well-studied mode of STAT3 activation, GPCRs such as S1PR1 have been shown to promote STAT3 activation by directly binding to JAKs (28). Intriguingly, D2-like receptors have been shown to promote proliferation by activating tyrosine kinase activity (29, 30). This suggests a possible connection between JAK/STAT/IL-6 and the effects of DRD2-targeting antipsychotics on cancer cells.

Whereas high STAT3 activity is associated with more aggressive cancers, triple-negative breast cancers (TNBCs) in particular are associated with high STAT3 activity (20, 23). Previous work from our lab has also shown that IL-6 is a critical factor for the maintenance of tumor initiating cells in triple-negative breast cancer cell lines (TNBCLs) (31). Triple-negative breast cancer is a subtype of breast cancer that lacks the expression of the HER2, estrogen, and progesterone receptors. Consequently, there are no targeted therapies for TNBC, which has a poor prognosis compared with other breast cancer subtypes (32).

In this study, we show that the DRD2-targeting drug thioridazine inhibits the self-renewal of some TNBCLs, but not others. At higher concentrations, thioridazine can induce a G1 arrest and block cell viability in all tested TNBCLs. We demonstrate that the inhibition of self-renewal depends on DRD2 and activated STAT3. However, the strong reduction in cell viability is not only independent of STAT3 activity, but also independent of DRD2.

Results
Thioridazine reduces cell viability of all tested TNBCLs, but inhibits self-renewal of some TNBCLs

Thioridazine, a DRD2 inhibitor, has been reported to decrease cancer cell proliferation and stemness in leukemia, glioblastoma, and colorectal, lung, and breast cancer cell lines (9–12, 14, 15). DRD2 mRNA expression across different breast cancer subtypes was assessed using TCGA data. It is interesting to note the wide range in variability of DRD2 expression in all tumor subtypes (Fig. S1). Overall, basal-like and lobular tumors had highest average expression of DRD2 compared with other breast cancer subtypes (Fig. S1). Because basal-like tumors are predominantly triple-negative, the effects of thioridazine were assessed on a panel of triple-negative cell lines (33). Although high expression was also observed in lobular breast cancer, our group has previously studied the self-renewal of triple-negative breast cancers (31), so we studied the effects of DRD2-targeted antipsychotics in TNBCLs. To test whether thioridazine has effects on the self-renewal of TNBCLs, the in vitro tumorsphere assay was used. Six triple-negative cell lines were treated with DMSO, 1 μM, 2 μM, or 5 μM thioridazine once and were cultured for 7 days before the number of spheres was counted. Interestingly, some TNBCLs (SUM149, HCC1143, HCC1937) were found to be sensitive where thioridazine caused a dose-dependent decrease in tumorsphere number; whereas others (SUM159, MDA-MB-231, HCC38) were resistant, showing no significant decrease in tumorsphere numbers at these concentrations of thioridazine (Fig. 1A). To more stringently test whether thioridazine inhibits self-renewal, secondary tumorsphere formation of SUM149 and SUM159 cells was tested in the presence of thioridazine. Indeed, thioridazine treatment inhibits secondary tumorsphere formation of SUM149 cells, but not of SUM159 cells (Fig. 1B). Because thioridazine is an inhibitor of DRD2, the observed differences in sensitivity were predicted to arise from expression of DRD2. Although HCC1143 cells have abundant expression of DRD2 compared with the other TNBCLs, DRD2 mRNA expression did not correlate with whether thioridazine will inhibit sphere formation (Fig. 1C). Perhaps thioridazine inhibits sphere formation in SUM149 cells but not SUM159 cells because DRD2 is more highly expressed in the sphere-forming cells in the SUM149 cell line. To test this, DRD2 mRNA expression was measured in tumorspheres relative to adherently grown SUM149 and SUM159 cells. Indeed, DRD2 is expressed more highly in SUM149 tumorspheres than in adherently grown cells (Fig. S2). However, it is also expressed more highly in SUM159 tumorspheres than in adherently grown cells (Fig. S2). This suggests that the lack of response in SUM159 tumorspheres is not because of low or absent expression of the receptor. We are unable to specifically detect DRD2 protein by Western blotting as antibodies against dopamine receptors, as is the case with some other GPCRs, are known to be nonspecific (34, 35).

A decrease in tumorsphere formation may be caused by inhibited self-renewal, or indirectly via reduced cell proliferation or increased death. In this regard, thioridazine has been reported to decrease cell viability in a number of cancer cells (9–12). To test the effects of thioridazine on adherently grown TNBCLs, cell viability was measured by detecting ATP abundance after 72 h of thioridazine treatment. In agreement with studies on other cancer cell lines, thioridazine dramatically reduced cell viability in TNBCLs at higher doses (Fig. 1D). Interestingly, there is only a 3-fold range in the IC50 of thioridazine across all six TNBCLs tested (Table 1). We confirmed these results on SUM149 and SUM159 cells by counting cell numbers after 72 h of thioridazine exposure. Both the SUM149 cells and the SUM159 cells show reduced cell numbers when treated with 2 μM or 5 μM, but not 1 μM thioridazine (Fig. 1, E and F). Fewer SUM149 cells are counted after 2–4 days of 5 μM thioridazine (Fig. 1E). On the other hand, there are SUM159 cells still growing when treated with 5 μM thioridazine (Fig. 1F). It is interesting to note that 1 μM thioridazine inhibits tumorsphere formation in SUM149 cells, even though cell viability is unaffected. This suggests that at 1 μM thioridazine has a specific effect on self-renewal in this cell line. On the other hand, doses as high as 5 μM thioridazine did not affect tumorsphere number in SUM159 cells, but it does strongly, but not completely, inhibit proliferation. This suggests that thioridazine preferentially targets nonsphere-forming cells in this cell line.

To determine whether thioridazine leads to induction of apoptosis, the proportion of sub-G1 cells was analyzed in response to thioridazine treatment. 5 μM thioridazine treatment modestly increased the proportion of sub-G1 SUM149 cells...
Accordingly, a slight induction of caspase 3/7 activity and PARP-1 cleavage was observed (Fig. 2, B and C). These data indicate that whereas apoptosis increases modestly upon 5 \( \mu \)M thioridazine treatment, the loss in cell viability is primarily nonapoptotic at this concentration.

ONC201 is a novel compound known to strongly induce apoptosis in many different cancer cell types including colorectal, acute myeloid leukemia, and breast cancer cells (36–38). ONC201 was originally discovered for its ability to induce apoptosis by inducing TNF-related apoptosis-inducing ligand (TRAIL). ONC201 treatment inhibits AKT, which releases Foxo3a to the nucleus, and Foxo3a induces the transcription of TRAIL (36). We tested whether thioridazine may work via this mechanism. Although thioridazine does dose-dependently inhibit AKT (Fig. S3), an increase in nuclear Foxo3a is not observed, nor is there a significant increase in TRAIL production (Fig. S3). Therefore, although thioridazine does

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**Table 1**

| IC\(_{50}\) of thioridazine in six TNBCLs | SUM149 | HCC1143 | HCC1937 | SUM159 | HCC38 | MDA-MB-231 |
|----------------------------------------|--------|---------|---------|--------|-------|-------------|
| Thioridazine IC\(_{50}\) (\( \mu \)M) | 2.319  | 4.276   | 6.082   | 3.517  | 5.849 | 3.396       |

(Fig. 2A). Accordingly, a slight induction of caspase 3/7 activity and PARP-1 cleavage was observed (Fig. 2, B and C). These data indicate that whereas apoptosis increases modestly upon 5 \( \mu \)M thioridazine treatment, the loss in cell viability is primarily nonapoptotic at this concentration.

ONC201 is a novel compound known to strongly induce apoptosis in many different cancer cell types including colorectal, acute myeloid leukemia, and breast cancer cells (36–38). ONC201 was also a DRD2 antagonist, like thioridazine (39), and
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![Figure 2. Low doses of thioridazine do not induce apoptosis.](image)

A. SUM149 cells were treated with thioridazine at the indicated concentrations for 48 h. Cells were stained with propidium iodide and analyzed by flow cytometry to determine cell-cycle stage. B. SUM149 cells were treated with the indicated concentrations of thioridazine for 24 h, and apoptosis was measured using Caspase-Glo 3/7 Assay. C. SUM149 cells were treated with DMSO, 1 μM, 2 μM, or 5 μM thioridazine and cultured for 24 h. Cells were then harvested and full length and cleaved PARP-1 abundance was measured by Western blotting. All experiments were performed with three biological replicates. Significance for A was measured using a two-sample t test, and significance for B was measured using a one-sample t test. Error bars represent S.D., *p < 0.05; **p < 0.01.

Thioridazine induces cell-cycle arrest

To address whether thioridazine causes a cell-cycle defect, the cell-cycle distribution of SUM149 cells was assessed by flow cytometry after propidium iodide staining in cells that were treated with increasing doses of thioridazine for 48 h. An increase in the proportion of G0/G1 cells was observed when SUM149 cells were treated with 5 μM thioridazine (Fig. 3, A and B). Changes in proteins involved in S-phase entry were assessed by Western blotting in response to thioridazine treatment. Interestingly, an increase in the CDK inhibitor CDKN1A/p21 was observed after 4 h. Further, a decrease in CCND1/cyclin D1 and CDK4 was observed as little as 1 h (Fig. 3C). These data indicate that 5 μM thioridazine treatment blocks the G1-S phase transition, leading to a cell-cycle-arrest.

Thioridazine inhibits STAT3 activity

IL-6 is a pro-inflammatory cytokine known to promote tumor growth (40–43). Previous work from our group showed that IL-6 promotes tumor-imitating cells in TNBCLs (31), and it has been shown to be a part of an IL-6/STAT3 feed-forward loop that promotes resistance to trastuzumab in Her2+ breast cancer cells (26). We first measured IL-6 mRNA abundance in all six TNBCLs used in the tumorsphere assay. Interestingly, cell lines that were sensitive to thioridazine in the tumorsphere assay expressed more IL-6 mRNA (Fig. 4A). To test whether thioridazine treatment affects IL-6 expression, SUM149 cells were treated with thioridazine for 1–8 h, total RNA was isolated, and IL-6 mRNA abundance was measured by qPCR. Thioridazine induced a rapid decrease in IL-6 transcript abundance, and that suppression was sustained for at least 8 h (Fig. 4B). Secreted IL-6 protein was measured by ELISA 4 h after treatment with thioridazine. A dose-dependent decrease of soluble IL-6 in the medium was observed (Fig. 4C). IL-6 is a target gene of the STAT3 pathway and can also form a feed-forward loop by activating STAT3. For these reasons we investigated whether thioridazine treatment can inhibit STAT3 activation. To do this, we measured phosphorylation of STAT3 at Tyr-705, which is important for STAT3 dimerization and transcriptional activity (24). Thioridazine treatment caused a dose-dependent decrease in the phosphorylation of STAT3 at Tyr-705 in SUM149 cells, but not SUM159 cells (Fig. 4D). Inactive STAT3 is normally excluded from the nucleus, but translocates to the nucleus upon pathway activation. We tested whether SUM149 cells have nuclear STAT3, and whether thioridazine causes a decrease. Indeed, total nuclear STAT3, as well as pTyr-705–STAT3 decreased upon thioridazine treatment (Fig. 4E and F). Together, these data show that thioridazine inhibits STAT3 activation and downstream IL-6 transcription in SUM149 cells.

Thioridazine requires STAT3 to inhibit self-renewal, but not proliferation or survival

Having shown that thioridazine inhibits STAT3, we tested whether STAT3 is required for the ability of thioridazine to inhibit self-renewal and proliferation of SUM149 cells. First, we tested whether STAT3 is required for thioridazine-mediated inhibition of self-renewal. To do this, SUM149 cells were transfected with siControl or siSTAT3. Then they were cultured in a tumorsphere assay and treated with DMSO or 1 μM thioridazine and the number of spheres formed were counted after 1 week. As expected from previous results, thioridazine caused a reduction in sphere formation (Fig. 5A). Knockdown of STAT3 also reduced tumorsphere formation, consistent with its reported role in maintaining cancer cell self-renewal (Fig. 5A). Interestingly, 1 μM thioridazine did not cause an additional decrease in sphere formation in the siSTAT3-treated SUM149 cells (Fig. 5A). This suggests that thioridazine inhibits self-renewal in these cells by inhibiting STAT3 activity. Because thioridazine reduces IL-6 production, which itself also promotes STAT3 activation, we tested whether addition of recombinant IL-6 (rIL-6) could rescue tumorsphere formation. Indeed, rIL-6 treatment increased tumorsphere number in DMSO-treated SUM149 cells as well as it does in thioridazine-treated SUM149 cells (Fig. 5B). To confirm that IL-6 inhibition is downstream of thioridazine, we tested if rIL-6 treatment can induce pTyr-705–STAT3 in the presence of thioridazine. Indeed, rIL-6 induced...
pTyr-705–STAT3 in control-treated SUM149 cells and in thioridazine-treated SUM149 cells (Fig. 5C). Thioridazine also caused a reduction in cell viability at high concentrations. To test whether STAT3 inhibition is also required for thioridazine-induced inhibition of proliferation and cell viability, siControl- and siSTAT3-treated SUM149 cells were treated with 1 μM, 2 μM, or 5 μM thioridazine and cultured adherently for 72 h before the number of remaining cells were counted. Interestingly, siSTAT3 treatment had no impact on the proliferation of SUM149 cells, and thioridazine inhibited proliferation similarly in siControl- and siSTAT3-treated cells (Fig. 5D). These data demonstrate that whereas thioridazine inhibits self-renewal in
SUM149 cells via STAT3 inhibition, the proliferation and cell viability defects are not mediated by STAT3 inhibition.

**DRD2 promotes sphere formation in SUM149 cells**

To test unique functions for DRD2 in TNBCLs, we first measured tumorsphere formation in SUM149 and SUM159 cells treated with siControl or siDRD2. Interestingly, siDRD2 treatment reduced tumorsphere formation in SUM149 cells, but not in SUM159 cells (Fig. 6, A and B), consistent with the effects of thioridazine on tumorsphere number in these cells. The magnitude of DRD2 knockdown was confirmed by qPCR (Fig. S4). To confirm that this decrease in sphere formation was not because of an off-target effect of the siRNA, SUM149 cell lines stably expressing shRNA targeting DRD2 were generated, and DRD2 knockdown was confirmed by qPCR (Fig. S4). Again, knockdown of DRD2 decreased tumorsphere formation in SUM149 cells (Fig. 6C). To further confirm the specificity of knockdown results, DRD2-CRISPR cells were generated and tumorsphere formation was determined. A decrease in DRD2 mRNA was observed in DRD2-CRISPR cells (Fig. S4), and presence of mutations at the cut site were also confirmed (Fig. S5). Indeed, the CRISPR-DRD2 cells also formed fewer tumorspheres than control cells (Fig. 6D). To more stringently test whether DRD2 promotes self-renewal in SUM149 cells, DRD2-CRISPR spheres were dissociated and cultured again as secondary tumorspheres. Again, a decrease in tumorsphere formation was observed, although more modest than primary tumorsphere formation (Fig. 6E).

Figure 4. Thioridazine inhibits STAT3 activity and IL-6 production. A, quantitative PCR analysis of relative IL-6 mRNA abundance in six TNBCLs. The relative expression of each cell line is normalized to SUM149 expression. B, SUM149 cells were treated with 5 μM thioridazine for the indicated time points, total RNA was isolated, and IL-6 mRNA abundance was measured by quantitative PCR. C, the medium of SUM149 cells was replaced with fresh medium containing either DMSO or the indicated concentration of thioridazine. After 4 h, the media were harvested and secreted IL-6 protein was measured by ELISA. D, SUM149 and SUM159 cells were treated with DMSO, 1 μM, 2 μM, 5 μM, or 10 μM thioridazine for 1 h. Protein lysates were harvested and abundance of pTyr-705–STAT3 was measured by Western blotting. E, SUM149 cells were treated with 5 μM thioridazine for the indicated time points. Nuclear extracts were made and STAT3 abundance was measured by Western blotting. Histone H3 was used to measure nuclear isolation quality and loading, and tubulin was used to measure cytoplasmic purity. F, quantification of nuclear pTyr-705–STAT3 and total STAT3. The quantifications are the -fold change of relative pTyr-705–STAT3 and STAT3 normalized to H3. All experiments were performed with three biological replicates. Significance for A is measured using a two-sample t test, significance for other experiments was measured using a one-sample t test. Error bars represent S.D. *, p < 0.05; ***, p < 0.01.
Indeed, chlorpromazine treatment inhibited sphere formation in a dose-dependent manner (Fig. 6F). To further test if DRD2 promotes self-renewal in SUM149 cells, a highly specific DRD2 inhibitor amisulpride was used (44). Treatment with as little as 500 nM amisulpride caused a modest, yet significant decrease in tumorsphere formation in SUM149 cells (Fig. 6G). Interestingly, the reduction in tumorsphere formation induced by 1 μM amisulpride is similar to that induced by 1 μM thioridazine or chlorpromazine. This suggests that specifically targeting DRD2 affects self-renewal, but not proliferation (see below and “Discussion”). Tumorsphere formation in response to a well-characterized DRD2/3-specific agonist quinpirole was also tested, which caused an increase in tumorsphere formation (Fig. 6H). These data confirm that DRD2 promotes self-renewal in the SUM149 cell line.

**DRD2 promotes STAT3 activation**

Because DRD2 promotes tumorsphere formation in SUM149 cells, and thioridazine inhibits tumorsphere formation via STAT3 inhibition, we tested whether DRD2 itself regulates STAT3 activation. To do this we first used the DRD2 antagonist chlorpromazine and observed its effect on STAT3 phosphorylation in SUM149 cells. Indeed, chlorpromazine decreased pTyr-705–STAT3 (Fig. 7A). Additionally, amisulpride, the highly specific DRD2/3 inhibitor also decreased pTyr-705–STAT3 (Fig. 7B). We were unable to observe decreased pTyr-705–STAT3 when DRD2 was knocked down with siRNA or shRNA (Fig. S6). This may be because of compensatory mechanisms of STAT3 activation that arise within the hours or days after knockdown. To directly test if DRD2 can promote STAT3 activation, we overexpressed DRD2 in SUM159 cells. As shown earlier, thioridazine does not decrease pTyr-705–STAT3 in these cells (Fig. 4D). Interestingly, DRD2 overexpression increased pTyr-705–STAT3 in SUM159 cells (Fig. 7C), and most importantly, the increase in pTyr-705–STAT3 was sensitive to thioridazine treatment (Fig. 7C). Because 1 μM thioridazine reduces tumorsphere formation via STAT3 in SUM149 cells but very little pTyr-705–STAT3 decrease is observed when SUM149 cells are treated with 1 μM thioridazine (Fig. 4D), we tested whether 1 μM thioridazine could reduce pTyr-705–STAT3 in SUM149 cells cultured as spheres. Indeed, a decrease in pTyr-705–STAT3 was observed in SUM149 spheres treated with 1 μM thioridazine (Fig. 7D). Further, we show that similar to thioridazine treatment, rIL-6 treatment rescued tumorsphere number in shDRD2 SUM149 cells (Fig. 7E). Although pTyr-705–STAT3 is not decreased in shDRD2 cells, rIL-6 increased pTyr-705–STAT3 (Fig. 7F). This result shows that DRD2 is not required for the effects of IL-6, and that IL-6 is downstream of DRD2/STAT3 in these cells. These data confirm that DRD2 can promote STAT3 activity in SUM149 cells, and that thioridazine targets DRD2 to inhibit STAT3 activity.
Thioridazine inhibits self-renewal, but not cell viability, via DRD2 inhibition

It is still unknown whether thioridazine reduces self-renewal and cell proliferation by inhibiting DRD2, or if those functions result from its activities on other receptors. To test whether the self-renewal inhibition by thioridazine results from DRD2 inhibition, the tumorsphere assay was used. SUM149 cells treated with siControl or siDRD2 were also treated with DMSO or 1/10IC50 thioridazine. As expected, both siDRD2 and thioridazine treatment alone caused a reduction in tumorsphere formation. To confirm this, the same experiment was repeated, but with DRD2-CRISPR SUM149 cells. Although 1 μM thioridazine decreased tumorsphere formation in control cells, it had no effect on DRD2-CRISPR cells (Fig. 8B). The ability of thioridazine to inhibit self-renewal depends on DRD2. To do this, we first observed the growth of SUM149 DRD2-CRISPR cells by counting the number of cells 72 h after seeding. Interestingly, a decrease in cell number in DRD2-CRISPR cells was not observed (Fig. 8C). We also counted cell numbers after treatment with the DRD2-specific inhibitor amisulpride, and no proliferation inhibition was detected at any dose tested (Fig. 8D). This indicates that DRD2
does not support cell proliferation in SUM149 cells. Additionally, we measured whether DRD2 is required for thioridazine to inhibit proliferation in SUM149 cells. To do this, control or DRD2-CRISPR cells were treated with DMSO or thioridazine for 72 h and then cells were counted. Thioridazine induced the same dose-dependent decrease in proliferation in DRD2-CRISPR cells as in control cells (Fig. 8E). These data show that although thioridazine inhibits sphere formation and self-renewal via DRD2 inhibition, it inhibits proliferation and cell viability by a different mechanism.

Discussion

Antipsychotics that target DRD2 have been shown to inhibit proliferation and self-renewal as well as induce apoptosis in leukemia and in lung, colon, and brain cancer cells (9–13). The effects of these compounds are generally assumed to be mediated by DRD2 inhibition. Notably, these studies only report strong effects when thioridazine is used at 10–20 μM (9–12). At these concentrations, thioridazine may have effects not mediated by DRD2 inhibition, and determining which effects of antipsychotic treatment are DRD2-dependent or independent is an important distinction to make to further identify drug targets in cancer. In addition, the mechanisms that mediate the antitu-
mor effects of these compounds remain largely unknown.

In this study, we demonstrate that thioridazine blocks the self-renewal of several TNBCLs, although the self-renewal of other TNBCLs is unaffected (Fig. 1, A and B). Importantly, we show that thioridazine inhibits self-renewal via DRD2 (Fig. 8). In addition, we demonstrate that additional DRD2-targeting antipsychotics also inhibit self-renewal (Fig. 6). We also show that thioridazine induces a G1 arrest and dramatically decreases proliferation (Figs. 1 and 3). These proliferation effects of thioridazine are not dependent on DRD2 (Fig. 8D). Further, we demonstrate that DRD2 promotes self-renewal by regulating STAT3/IL-6 activity in SUM149 cells. STAT3, similar to DRD2, does not support proliferation and cell viability, but instead primarily supports self-renewal (Fig. 5). Based on these data, we developed a model where DRD2 supports STAT3 activation to maintain a STAT3/IL-6 feed-forward loop and persistent STAT3 activation (Fig. 8F).

Regarding the effects of thioridazine on self-renewal, we show that concentrations as low as 1 μM thioridazine are capable of decreasing tumorsphere formation in SUM149 cells (Fig. 1, A and B), and that proliferation and cell viability are unaffected at those doses (Fig. 1, E and F). This suggests that effects on self-renewal are not confounded by effects on cell viability at that dose. Furthermore, the DRD2/3-specific antagonist amisulpride blocks sphere formation to a similar degree as 1 μM thioridazine (Figs. 6G and 1B), even though it does not inhibit proliferation (Fig. 8D). This further supports that DRD2 promotes self-renewal in SUM149 cells, but the proliferation inhi-
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Figure 8. Thioridazine inhibits self-renewal, but not cell viability via DRD2 inhibition. A, SUM149 cells were transfected with either siControl or siDRD2 and then cultured in a tumorsphere assay. Each group was also treated with DMSO or 1 μM thioridazine and tumorspheres were counted after 1 week. B, control and DRD2-CRISPR SUM149 cells were cultured in a tumorsphere assay, treated with DMSO or 1 μM thioridazine and the number of spheres formed was counted after 1 week. C, SUM149 DRD2-CRISPR cells were seeded and the number of cells were counted after 72 h. D, SUM149 cells were seeded and treated with the indicated concentration of amisulpride. Cells were then counted after 72 h. E, SUM149 control or DRD2-CRISPR cells were treated with DMSO 1 μM, 2 μM, or 5 μM thioridazine and cells were counted after 72 h. F, model figure describing mechanism of thioridazine effects on TNBCs. All experiments were performed with three biological replicates. Significance for E is measured using a two-sample t test; significance for other experiments was measured using a one-sample t test. Error bars represent S.D. *, p < 0.05.

Thioridazine inhibits self-renewal and proliferation is DRD2-independent. In contrast, in SUM159 cells doses of thioridazine as high as 5 μM do not affect tumorsphere formation (Fig. 1B), although cell viability is dramatically decreased (Fig. 1, D–F). This indicates that whereas thioridazine can reduce the proliferation and viability of the bulk of SUM159 cells, it is unable to inhibit the activity of sphere-forming cells at these concentrations. Together, our data show that in certain cells (SUM149) DRD2 supports sphere-forming cells, and that this activity can be targeted with thioridazine, when used at 1 μM. As increasing doses of thioridazine are used (5 μM), a G1 arrest and loss of cell viability independent of DRD2 activity occurs. In other cells (SUM159), thioridazine cannot inhibit self-renewal via DRD2/STAT3 in sphere-forming cells. However, thioridazine can still inhibit the proliferation of the bulk of cells at 5 μM, but the sphere-forming cells are more resistant than the bulk of the cells. Uncovering the cell types in which this DRD2/STAT3/IL-6 pathway can be targeted would be an important step in determining which tumors can be most effectively treated with thioridazine or other DRD2-targeting antipsychotics.

The STAT3/IL-6 activation loop is an established cancer promoting pathway. High systemic IL-6 correlates with poorer prognosis and advanced tumor stage in breast cancer patients (42), and STAT3 is known to promote breast cancer survival, invasion, drug resistance, and stemness (17, 23, 25, 27). For these reasons, there has been significant research into targeting the STAT3/IL-6 pathway. However, STAT3, like many transcription factors, has proven difficult to specifically target with small compounds. Although antibodies that target both IL-6 and the IL-6 receptor have been developed and have had major impacts in inflammation-related diseases, translation into cancer therapies has been slow (45). Mechanisms whereby inhibiting DRD2 has anti-cancer effects remain largely unknown. Here we demonstrate that DRD2 promotes STAT3 activation and that thioridazine and other DRD2-targeting compounds block that activity in SUM149 cells. Using tumorsphere assays, we also show that STAT3 is required for DRD2 to promote self-renewal, and thioridazine does not block self-renewal in the absence of STAT3 in SUM149 cells. We observed the strongest decrease of pTyr-705–STAT3 at 5–10 μM thioridazine. At these doses loss of cell viability is observed, not just self-renewal defects. However, the Western blotting was performed after 1 h of treatment from cells grown adherently. We also show that addition of recombinant IL-6 rescues tumorsphere number of thioridazine-treated and shDRD2 SUM149 cells. IL-6 treatment is also still able to increase pTyr-705–STAT3, even with thioridazine treatment or in DRD2 knock-down cells. This indicates that IL-6 is downstream of DRD2/STAT3, and that DRD2 likely supports STAT3 activity, and IL-6 is subsequently promoted via increased STAT3 activity.

Notably, we demonstrated that thioridazine induces a G1 arrest and a decrease in cell proliferation that is independent of...
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DRD2/STAT3 in SUM149 cells. We also determined that 5 μM thioridazine causes a reduction in proliferation without a dramatic increase in apoptosis. (Figs. 1 and 2). We also show using DRD2-CRISPR cells and the highly specific DRD2/3 inhibitor amisulpride that DRD2 does not support proliferation and viability in the bulk of tumor cells (Fig. 8, C and D). Importantly, we demonstrate that thioridazine is not dependent on the presence of DRD2 to inhibit proliferation in SUM149 cells (Fig. 8E). This is supported by work from Kline et al. (46), which showed that a novel DRD2 antagonist, ONC201, can lead to reduced cell proliferation in both control and DRD2 knockout colorectal cancer cells. The mechanisms by which these DRD2-independent effects occur are unknown, but they may offer insight into interesting new drug targets for TNBC.

The origin of an activating ligand in cancer, if any, for DRD2 is still unknown. The catecholamine dopamine is generally considered the primary activator for all dopamine receptors, including DRD2 (2). It is unknown what the potential source of dopamine could be in the breast cancer milieu. Interestingly, T-cells, B-cells, dendritic cells, and macrophages have all been shown to express dopamine receptors (6, 47). These cells have been shown to be capable of dopamine synthesis (6, 48–51), and dopamine has been demonstrated to either promote or inhibit their activity (6, 47, 49, 50, 52). It is possible that tumor infiltrating immune cells could secrete dopamine and other catecholamines and support tumor growth and/or suppress antitumor immunity. However, our study is based on cell lines grown in vitro. So even if infiltrating immune cells are a source of dopamine in vivo, dopamine receptors have cancer cell line autonomous effects. Whether this is a result of intrinsic, ligand-independent, activity of dopamine receptors or whether cancer cells themselves maybe secreting dopamine is unclear. Additionally, there is evidence that proteins, such as the noncanonical Wnt5a, can also activate DRD2 (53). The implications of this are intriguing, but mostly unexplored.

It is also unknown which cell types are most sensitive to thioridazine and other DRD2-targeting compounds. We show that the self-renewal of several TNBCs (SUM149, HCC1143, HCC1937) is strongly inhibited by thioridazine, whereas the self-renewal of three other TNBCs (SUM159, MDA-MB-231, HCC38) is unaffected by thioridazine (Fig. 1B). The inhibition of self-renewal caused by 1 μM thioridazine depends on DRD2 (Fig. 8, A and B). Interestingly, we also show that although the TNBCs in which thioridazine inhibits self-renewal do not express more DRD2 mRNA (Fig. 1D), they express more IL-6 mRNA (Fig. 4A). Further, thioridazine inhibits STAT3 activation in SUM149 cells, but not SUM159 cells (Fig. 4D), and STAT3 is required for thioridazine to inhibit self-renewal in SUM149 cells (Fig. 5). These findings suggest that in some cell types, as in SUM149 cells, the DRD2 promotes STAT3/IL-6 activity to support self-renewal, whereas in other cell types, as in SUM159 cells, STAT3 activity is not supported by DRD2 and therefore DRD2 inhibition does not block self-renewal. The effects of thioridazine on STAT3 activation and tumor growth of the murine 4T1 triple-negative breast cancer model have already been tested. Although 4T1 tumor growth is suppressed by thioridazine treatment, STAT3 phosphorylation is unaffected (14). This is similar to the effects of thioridazine on the TNBC SUM159. The connection between DRD2 activity and STAT3/IL-6 needs to be explored further to determine the cell types in which targeting DRD2 is likely to lead to a specific inhibition of self-renewal.

We show that the effects of thioridazine on proliferation and cell viability are independent of DRD2. Finding the receptors responsible for these effects may be important but complicated, as thioridazine is known to bind other GPCRs (44). Interestingly, antagonism of serotonergic receptors has been shown to reduce breast cancer growth (54). Important roles of adrenergic receptors in promoting progression and angiogenesis of prostate cancer have also been recently reported (55, 56). Additionally, a recent study showed that treatment of glioblastoma cells with DRD4 antagonists inhibited autophagy, led to a G1 arrest, and ultimately induced apoptosis (57). This implicates DRD4 as potentially regulating cancer cell survival in glioblastoma. However, mechanisms by which antipsychotics like thioridazine can achieve such dramatic effects across so many cell types remain elusive.

This study has shown that DRD2 can promote STAT3 activity and self-renewal, and antipsychotics that preferentially target DRD2 can inhibit this pathway in SUM149 cells. Interestingly, these antipsychotics also have strong effects on cell proliferation and cell viability that are not a result of DRD2 inhibition broadly, in all TNBCs tested. Thioridazine is generally safe and has shown to be potent at reducing cancer cell viability, and there may be potential therapeutic benefit to using thioridazine or other DRD2-targeting compounds in the cancer clinic.

Experimental procedures

Cell culture and reagents

SUM149 and SUM159 cells were maintained in HuMEC medium (Gibco) with 5% FBS. MDA-MB-231 cells were maintained in DMEM (Gibco) with 10% FBS. HCC38, HCC1143, and HCC1937 cells were maintained in RPMI 1640 with 10% FBS. Thioridazine, chlorpromazine, forskolin, amisulpride, and quinpirole were obtained from Sigma-Aldrich. All antibodies were obtained from Cell Signaling Technologies (Danvers, MA). pLENTI6-V5 vector was obtained from the CCSB-Broad Leiden, MN). All siRNAs were SMARTpool siGENOME from Dharmacon (Lafayette, CO). DRD2 overexpression plasmid in pLENT16-V5 vector was obtained from the CCSB-Broad Lentiviral Expression Collection (ORF) (58).

Stable shDRD2 and DRD2-CRISPR cell lines

DRD2-targeting shRNA constructs (TRCN0000011342 and TRCN0000011343) in pLKO.1 vector backbone were obtained from the Open Biosystems TRC1 shRNA library. The plasmids were transfected into HEK293T cells using FuGene HD (Promega, Madison, WI). The supernatant was collected then filtered using 0.4 μm filters. The resulting liquid was then concentrated to ~150 μl using Amicon Ultra-15 Ultrace-100
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(EMD Millipore, Billerica, MA). The virus concentrate was then added to SUM149 cell growing in 10 ml of HuMEC medium before puromycin selection. lentCRISPR v2 was a gift from Feng Zhang (Addgene plasmid no. 52961). Genomic sequences to target were identified using predictive software from the Zhang laboratory. Sequences of the primers to clone the DRD2 CRISPR constructs are as follows: 5′-CTGCCTTATTGAGTCCGAAG-3′ and 5′-GTAGGCGGTATTGATCAGCA-3′. Lentivirus was made as stated previously. Pooled CRISPR mutants that survived puromycin selection were used for experiments.

Immunoblots, cell fractionation, and immunoprecipitation

Immunoblots and immunoprecipitations were performed as described previously (59), except Mini-PROTEAN TGX SDS-PAGE gels (Bio-Rad) and Clarity ECL (Bio-Rad) were used. To obtain nuclear and cytoplasmic extracts, cells were lysed with CE buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% Nonidet P-40, 1 mM DTT, and protease and phosphatase inhibitor cocktails). Cytoplasmic extract was removed and the resulting nuclei were washed in CE buffer then lysed in NE buffer (62.5 mM Tris-HCl, 5% glycerol, 2% SDS, 5% β-mercaptoethanol). The lysates were then immunoblotted as described previously.

RNA isolation and quantitative PCR

Total RNA was isolated using TRIzol (Invitrogen) according to manufacturer’s protocol. iScript (Bio-Rad) was used to make cDNA. Quantitative PCR was performed with iTaq Probes Supermix (Bio-Rad) using an QuantStudio 6 (Applied Biosystems, Foster, CA) and analyzed using QuantStudio software to calculate RQ values relative to GUSB housekeeping gene.

Tumorsphere assay

Cells were trypsinized and resuspended into a single cell suspension and counted using a hemocytometer. 20,000 cells were resuspended in 2 ml of complete MammoCult Medium (Stemcell Technologies, Vancouver, BC, Canada) and plated into ultralow-adherence 6-well plates (Corning Inc., Corning, NY). After 1 week of culture, the spheres were collected and all spheres greater than 60 μm in diameter were counted according to manufacturer’s protocol (Stemcell Technologies). Secondary tumorsphere formation was assessed by culturing primary spheres as described; the resulting spheres were dissociated with trypsin and 20,000 cells were again plated in 2 ml of complete MammoCult into ultralow-adherence 6-well plates. Thioridazine treatments were applied once at the time of plating. Quinpirole, chlorpromazine, amisulpride, rIL-6, and anti-IL-6R antibody were applied daily. Experiments with siDRD2 were performed as described except only 200 cells were plated in 2 ml of MammoCult.

Transfections

Overexpression analysis was achieved by transfecting 10 μg of plasmid with 20 μl of FuGENE HD (Promega). siRNA transfections were achieved by using 40 nM siRNA and DharmaFECT 1 (Dharmacon) according to manufacturer’s protocol.

Proliferation, cell viability, and caspase assays

To assess proliferation, cells were plated in 24-well plates at 4000 cells per well in triplicate (SUM159), or 6500 cells per well in triplicate (SUM149). Cells were counted at indicated times using a hemocytometer. Cell viability assays were performed by plating 1000 cells in a 96-well plate in medium with the appropriate concentration of thioridazine. After 72 h, cells were treated with 100 μl of CellTiter-Glo (Promega) for 10 min. Luciferase activity was measured using a Synergy 2 Luminometer (BioTek, Winooski, VT). IC50 was determined using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). To assess caspase 3/7 activity, 2500 SUM149 cells were plated per well in a 96-well plate and treated with indicated concentrations of thioridazine for 4, 24, or 48 h. Assay was performed according to manufacturer’s protocol. Luciferase activity was measured using a Synergy 2 Luminometer (BioTek).

Cell-cycle analysis

SUM149 cells were treated with DMSO or thioridazine for 48 h and fixed in ice-cold 70% ethanol for 2 h at 4°C. The cells were resuspended in flow buffer (1× PBS, 10% FBS) and treated with 10 μg/ml RNase A for 30 min at 37°C. 5 μl of 1 mg/ml propidium iodide (Invitrogen) was added for 30 min at room temperature. Cells were then washed in flow buffer and filtered using a 40 μm filter (BD Biosciences). Data were acquired using a Cyan ADP Flow Cytometer (BD Biosciences) and analyzed using FloJo software (Ashland, OR).

ELISA

To measure soluble IL-6, fresh HuMEC medium with DMSO or thioridazine was added to SUM149 cells in 6-well plates. 4 h later, the medium was collected and centrifuged to remove insoluble material. Then the human IL-6 BD OptEIA kit was used to detect the IL-6 according to manufacturer’s protocol (BD Biosciences). To measure cAMP, cells in a 6-well plate were treated with 1 μM forskolin and either DMSO or quinpirole. Cells were additionally treated with 2 μM thioridazine where indicated. After a 15-min incubation, the cells were harvested and ELISA was performed according to manufacturer’s protocol using the cAMP Select ELISA Kit (Cayman Chemical, Ann Arbor, MI).

TCGA analysis

Expression of DRD2 across breast cancer subtypes was analyzed in the breast invasive carcinoma samples in the TCGA dataset. The subtype calls are from the PAM50 50-gene subtype analysis as described previously (60). A one-way analysis of variance (ANOVA) was performed to compare the gene expression values in multiple groups, which are displayed by boxplot.

Statistics

Statistical significance tests were done as described in each figure. GraphPad Prism 7 software (GraphPad Software) was
used for all graphs and significance tests unless otherwise noted.

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