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Dopamine Receptor D5 is a Modulator of Tumor Response to Dopamine Receptor D2 Antagonism

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Abstract

Purpose: Dopamine receptor D2 (DRD2) is a G protein-coupled receptor antagonized by ONC201, an anti-cancer small molecule in clinical trials for high grade gliomas and other malignancies. DRD5 is a dopamine receptor family member that oppose DRD2 signaling. We investigated the expression of these dopamine receptors in cancer and their influence on tumor cell sensitivity to ONC201.

Experimental Design: The Cancer Genome Atlas was used to determine DRD2/DRD5 expression broadly across human cancers. Cell viability assays were performed with ONC201 in >1,000 Genomic of Drug Sensitivity in Cancer and NCI60 cell lines. Immunohistochemistry staining of DRD2/DRD5 was performed in tissue microarrays and archival tumor tissues of glioblastoma patients treated with ONC201. Whole exome sequencing was performed in RKO

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cells with and without acquired ONC201 resistance. Wild-type and mutant DRD5 constructs were generated for overexpression studies.

Results: DRD2 overexpression broadly occurs across tumor types and is associated with a poor prognosis. Whole exome sequencing of cancer cells with acquired resistance to ONC201 revealed a de novo Q366R mutation in the DRD5 gene. Expression of Q366R DRD5 was sufficient to induce tumor cell apoptosis, consistent with a gain-of-function. DRD5 overexpression in glioblastoma cells enhanced DRD2/DRD5 heterodimers and DRD5 expression was inversely correlated with innate tumor cell sensitivity to ONC201. Investigation of archival tumor samples from recurrent glioblastoma patients treated with ONC201 revealed that low DRD5 expression was associated with relatively superior clinical outcomes.

Conclusions: These results implicate DRD5 as a negative regulator of DRD2 signaling and tumor sensitivity to ONC201 DRD2 antagonism.

Keywords

ONC201; imipridone; DRD2; DRD5; dopamine; GPCR; glioma; glioblastoma; cancer

Introduction

G protein-coupled receptors (GPCRs) are the largest superfamily of membrane receptors in humans. However, these receptors are underexploited therapeutic targets for oncology that control several signaling pathways that are critical for cancer, including the integrated stress response and Ras signaling [1]. Overexpression of the GPCR dopamine receptor D2 (DRD2) in cancer and anti-cancer effects of DRD2 antagonism via induction of the integrated stress response and inhibition of Akt/ERK signaling have been reported in a range of tumor types [2–5].

The imipridone class of anti-cancer compounds share a unique tri-heterocyclic core chemical structure [6] and selectively target GPCRs [7, 8]. ONC201 is the first imipridone to enter clinical trials [9] and is a selective antagonist of dopamine receptor D2 (DRD2) and D3 (DRD3). This compound has exhibited encouraging safety, pharmacokinetic, pharmacodynamic and efficacy profiles in Phase I/II trials, including sustained tumor regressions in advanced chemo-resistant cancers such as glioblastoma, endometrial cancer and mantle cell lymphoma [7, 10–12]. DRD2 antagonism by ONC201 results in activation of the integrated stress response [13, 14] and inactivation of Akt/ERK signaling that induces downstream DR5/TRAIL-mediated apoptosis in cancer cells and impairs cancer stem cell self-renewal [9, 15, 16].

We investigated the dysregulation of DRD2 in human cancer and its role in tumor response to ONC201 to uncover predictive biomarkers.

Materials and methods

Cell culture and reagents

ONC201-resistant RKO human colon cancer cells have been generated previously using increasing concentration gradient incubations [13]. All other cell lines were obtained from

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the American Type Culture Collection (ATCC) and cultured as per ATCC recommendations. Cells were authenticated every month by bioluminescence, growth and morphological observation. ONC201 was provided by Oncoceutics, Inc.

GPCR profiling

Experimental GPCR profiling was performed utilizing the PathHunter beta-arrestin enzyme fragment complementation (EFC) assay at DiscoverX as described previously [17, 18]. PathHunter cells were seeded in a total volume of 20 μL into white walled, 384-well microplates and incubated at 37°C for the appropriate time prior to testing. For agonist determination, cells were incubated with test compound to induce response. 5 μL of 5x test compound was added to cells and incubated at 37°C or room temperature for 90 or 180 min. For antagonist determination, cells were preincubated with test compound followed by agonist challenge at the EC80 concentration (5 μL of 5x sample was added to cells and incubated at 37°C or room temperature for 30 min and 5 μL of 6x EC80 agonist in assay buffer was added to the cells and incubated at 37°C or room temperature for 90 or 180 min). Final assay vehicle concentration was 1%. Assay signal was generated through a single addition of 12.5 or 15 μL (50% v/v) of PathHunter Detection reagent cocktail, followed by 1-h incubation at room temperature. Microplates were read following signal generation with a PerkinElmer EnvisionTM instrument for chemiluminescent signal detection. Compound activity was analyzed using CBIS data analysis suite (ChemInnovation, CA). GPCR panel was performed with 10 μM test compound.

Genomics of Drug Sensitivity in Cancer (GDSC) cell line screening

Cell viability assays were performed as previously described [19, 20] with >1,000 human cancer cell lines at 72 h post-ONC201 treatment to generate dose responses curves at concentrations from 78 nM up to 20 μM. Cell viability was determined using either a DNA dye (Syto60) or metabolic assay (Resazurin or CellTitre-Glo). Fluorescence intensity data from screening plates for each dose response curve is fitted using a multi-level fixed effect model [21] to generate IC50 and area under curve (AUC).

Propidium iodide staining and cell viability assays

Cells were treated, trypsinized, ethanol-fixed, stained with propidium iodide (Sigma) and analyzed by flow cytometry as previously described [9]. Cell viability was determined as described previously using the CellTitre-Glo reagent (Promega) [15].

Western blot

Western blotting was performed as described previously [8, 11, 18]. Briefly, lysates were prepared and evaluated with protein assay (Biorad). LDS sample buffer and reducing agent (Invitrogen) were added for SDS-PAGE. After transfer, primary and secondary antibody incubations were performed, and signal was detected using a chemiluminescent detection kit, followed by autoradiography.

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Immunohistochemistry

Immunohistochemistry assessment of DRD2 (sc-5303, 1:300) and DRD5 (HPA048930, 1:100) expression in paraffin-embedded formalin-fixed archival tumor tissue of patients on trial was performed using the automated Leica Bond Rx system followed by dehydration and mounting. Following US Biomax tissue microarrays were used: glioblastoma (GBM) (GL805B), neuroblastoma (MC602), pheochromocytoma (AD2081), medulloblastoma (BC17012c) and endometrial cancer (EMC961). Additionally, the BioChain FDA Standard Tissue Array (T8234701–1) was also used. Tissue microarray and patient archival tumor tissue slides stained for DRD2 and DRD5 were scanned (Aperio AT2 slide scanner) and loaded into image analysis software (Visiopharm VIS). Whole slide images were annotated by a pathologist to create regions of interest (ROI) delineating tumor area and to set thresholds specific for DRD2- and DRD5-positive immunolabeling. Individual algorithms were developed to detect and stratify DRD2 or DRD5 immunolabeling area for each TMA core or patient sample, respectively. Each algorithm identified the number of pixels stained positively for DRD2 or DRD5 within the tumor ROI. Positively-stained pixels, converted to area was quantified as a percentage of total tumor area. Following quantification, each sample was reviewed to confirm appropriate algorithm application and quantification. The pathologist assigned a low threshold to account for background signal and determine a baseline positive.

Culture of medulloblastoma cells from patient-derived xenograft (PDX)

NOD-SCID IL2R-gamma null (NSG) mice used for intracranial tumor transplantation were purchased from Jackson Labs. Mice were maintained in the animal facilities at the University of California San Diego (UCSD). All experiments were performed in accordance with national guidelines and regulations, and with the approval of the animal care and use committees at UCSD. Medulloblastoma PDX Med211-FH tumor cells, generated by the Olson lab, were thawed and orthotopically transplanted into the cerebellums of a NSG mice. About 6–8 weeks later once the mice showed signs of tumor burden, they was sacrificed and the tumors was removed. Tumors were then dissociated and resuspended in NeuroCult medium with proliferation supplement (STEMCELL Technologies) and plated at 10,000 cells/well in 25ul a 384 well plate for ONC201 treatment. Viable cell number in each well was determined using the CellTiter-Glo reagent (Promega) and read in an automated Envision plate reader (PerkinElmer) after 48 hour incubation.

Clinical and pathologic characteristics of patients treated with ONC201

We previously reported clinical outcomes and intratumoral DRD2 expression in a cohort of 17 recurrent GBM patients (9 male and 8 female) who were treated with ONC201 [11]. Intratumoral expression of DRD5 was determined using archival tumor tissue as described above and correlated with clinical outcomes. The median age of patients recruited was 57 years (range 22–74 years) with WHO (2007) Grade IV histologically confirmed diagnosis of GBM and median KPS of 90 (range 70–100). Patients were bevacizumab naïve, did not have known IDH1/2 tumor mutations, and were previously treated with temozolomide and radiotherapy. MGMT status of patients were: 2 methylated, 13 unmethylated and 2 unknown.

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Computational Analyses

For TCGA analyses, the individual survival scores of each included cancer type were scaled to a 0 to 1 scale according to the following formula: (Value – Min(Values for Cancer Type)) / (Max(Values for Cancer Type) – Min(Values for Cancer Type)). For each cancer type a z-score was calculated based on the log10(expression + 1). A Fisher's exact test was used to measure significance with an expression z-score cutoff of 1 and a normalized survival value cutoff of .5. All data was downloaded from TCGA. For the GBM cohort analysis, we performed a Likelihood Ratio Chi-square two-tailed test to examine the significance of these results at 455 days of follow-up.

The generalized linear model was performed on the GDSC efficacy testing data with the caret package and the R statistical programming language. Expression values of DRD1 – DRD5 in all cells were used to predict the overall efficacy of ONC201 in that same cell (measured by IC50). The normalized coefficient score was obtained by dividing the coefficient of each individual variable (DRD1–5) by the maximum of the absolute value of all 5 coefficients: $Coef_n/(Max(Abs(Coef_{DRD1\text{-}DRD5})$.

The loss-of-function (LoF) essentiality data was downloaded via Project Achilles. The shRNA LoF screening results were collected from Version 2.20.2 and pre-calculated DEMETER values were used as essentiality scores for DRD2. Drug efficacy scores for ONC201 were collected from the GDSC cell line sensitivity screening panel. The correlation between drug efficacy, AUC scores, and DRD2 DEMETER essentiality scores for brain cancer cell lines were evaluated using the Spearman Correlation test.

Results

DRD2 is overexpressed in human cancer

Investigation of RNA-seq data in The Cancer Genome Atlas (TCGA) revealed that DRD2 is expressed broadly amongst human cancers, however somatic mutations are infrequent (Fig. S1A–B). Comparing DRD2 expression in malignant versus corresponding normal tissues revealed selective overexpression of the receptor in numerous tumor types (Fig. 1A). Pooling multiple tumor types in TCGA that are annotated with survival outcomes, we observed that patients with high levels of DRD2 expression had relatively inferior overall survival (Fig. 1B). Immunohistochemical analysis of tissue microarrays corroborated TCGA observations that malignant DRD2 expression was highest in pheochromocytoma/paraganglioma (PCPG), GBM, neuroblastoma, medulloblastoma and endometrial cancer (Fig. 1C–D and Fig. S2A–D), which are tumor types that are sensitive to ONC201 in vitro (Fig. S3A).

DRD2 dysregulation in high grade gliomas and concordance with tumor response to ONC201

DRD2 exhibited significant essentiality scoring derived from large scale CRISPR screens [22] across numerous cancer cell lines associated with various primary sites of disease (Fig. 2A). Central nervous system cancers had the highest DRD2 gene essentiality scores, indicating that these cancer types are the most vulnerable to DRD2 antagonism among the

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panel. Given these observations and the activity of ONC201 in high grade glioma preclinical models and patients [9, 11, 23], we further investigated DRD2 expression in this tumor type. DRD2 was highly expressed relative to the other 4 dopamine receptors (Fig. 2B), however genetic aberrations were rare among the 273 evaluated GBM specimens: gene amplification in 3 specimens (1.1%), R219H mutation in 1 specimen (0.4%), and no gene copy loss (Fig. S1C). Patients with high DRD2 expression tended to have primary, rather than secondary, GBM (Fig. S1D). Patients who had relatively long overall survival (>2 years) exhibited low expression of DRD2 while patients with inferior survival had heterogeneous DRD2 expression, suggesting that low DRD2 expression is necessary, but not sufficient, for relatively long overall survival (Fig. 2C). A similar trend was observed in low grade glioma with disease-free survival (Fig. S1E).

A correlation between DRD2 mRNA and ONC201 GI50 was observed among a panel of 6 GBM cell lines in the NCI60 panel (Fig. 2D), but not other tumor types (Fig. S3B). Similarly, we found a significant concordance (cor = 0.57, p-value < 0.05) between a cell line's sensitivity to ONC201 within the Genomics of Drug Sensitivity in Cancer (GDSC) panel and cell line-specific DRD2 gene essentiality scores for brain cancers (Fig. 2E). Additionally, we observed a modest concordance between tumor type sensitivity to ONC201 in the GDCS panel and tumor type sensitivity to DRD2 RNAi by ATARiS scoring (Fig. S3C). Lymphoma, a tumor that ONC201 is being evaluated in a Phase I/II trial (NCT02420795)[7], exhibited the strongest sensitivity to both DRD2 RNAi by ATARiS scoring and ONC201 response by cell viability.

DRD5 is an inversely correlated predictive biomarker of ONC201 efficacy

To further evaluate the relative contribution of dopamine receptors (DRD1–5) to the efficacy of ONC201 among the GDSC panel, we used a generalized linear model to combine the expression of all five receptors into a single model. We then ranked the relative contribution of each dopamine receptor based on the coefficient value assigned to it by the generalized linear model (Fig. S3D). In accordance with our previous results we found that the strongest negative contributor was DRD2 – where a negative contribution denotes a decreased IC50 value as expression increases. Interestingly, we found that DRD5 had the highest positive score – indicating that low expression of DRD5 was correlated with enhanced sensitivity to ONC201. DRD5 is a G_s-coupled D1-like dopamine receptor that exhibits downstream signaling effects (e.g. cAMP production) that counteract D2-like receptors that are G_i-coupled, such as DRD2.

To further explore biomarkers of tumor response to ONC201, we performed whole exome sequencing of RKO cell line clones with and without acquired resistance to ONC201 that we previously generated [13]. Analysis of non-synonymous mutations revealed a consensus heterozygous Q366R mutation in the DRD5 gene that was exclusive to the resistant clones (Fig. 3A–B). Given that the sequenced cells were driven by complete and stable resistance to ONC201, we hypothesized that the acquired resistance clones evolved to be DRD2-independent due to stable inactivation of DRD2 signaling via DRD5. In accordance with Q366R acting as a mutation in DRD5 that enhances its ability to antagonize DRD2 signaling, Q366R DRD5 overexpression transiently induced tumor cell death to a greater

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extent than the wild-type gene (Fig. 3C). Dimerization of DRD2/DRD5 can occur in cells via electrostatic interactions between intracellular residues [24]. Overexpression of WT or Q366R DRD5 was sufficient to induce DRD2 oligomers that are presumed to be DRD2/DRD5 heterodimers (Fig. 3D). In accordance with the hypothesis that ONC201 sensitivity is associated with innately low DRD5 expression to facilitate DRD2 downstream signaling, overexpression of wild-type DRD5 prior to treatment conferred resistance to ONC201-mediated apoptosis (Fig. 3E). These results suggest that DRD5 is a direct negative regulator of DRD2 signaling that can influence innate tumor cell sensitivity to DRD2 antagonism by ONC201.

Interrogation of TCGA and tissue microarrays revealed heterogeneous malignant and normal tissue DRD5 expression that was generally lower in magnitude than DRD2 and exhibited a different spectrum of expression across tumor types (Fig. 3F and Fig. S2E). Somatic alterations were more common in the DRD5 gene compared to DRD2, in particular missense mutations, however the Q366R mutation was not found (Fig. S3E). These results indicate that the mechanisms of dysregulation in oncology may be distinct for these two dopamine receptors. A similar analysis of the clinical prognostic impact of DRD5 expression and clinical outcome did not yield a significant relationship (Fig. S3F), unlike DRD2, suggesting that the role of DRD5 in cancer may be limited to influencing response to DRD2 antagonism.

Exploring the influence of DRD5 on innate tumor cell sensitivity, we found that DRD5 mRNA levels are inversely correlated with ONC201 sensitivity in the NCI60 panel (Fig 3G and Fig. S3G–H). The hypothesis that DRD2+ DRD5- tumor cells are highly responsive to ONC201 held true in the GDSC panel (Fig. 3H). Thus, DRD5 is a negative regulator of DRD2 signaling that is associated with decreased tumor cell sensitivity to DRD2 antagonism by ONC201.

Given the influence of DRD5 on tumor cell sensitivity to DRD2 antagonism, we evaluated the impact of dopamine receptor selectivity on the efficacy of DRD2 antagonism in cancer cells. We found that DRD2-specific antagonism results in anti-cancer effects in vitro that are superior to concomitant antagonism of DRD2 and DRD5 (Fig. S4A–B). While ONC201 has been previously described to be highly selective for DRD2/DRD3 [25], antipsychotics such as haloperidol and chlorpromazine affect several dopamine receptors and other GPCRs (Table S1 and Fig. S4C–D) at concentrations required to kill cancer cells [2, 5]. Accordingly, ONC201 demonstrated a wide and superior therapeutic window in vitro (Table S2).

Intratumoral DRD5 expression is associated with clinical outcomes in ONC201-treated recurrent glioblastoma patients

We previously reported that DRD2 was expressed in archival tumor specimens of recurrent GBM patients who were treated with ONC201 [11]. Based on the observation that DRD5 is a predictive biomarker for ONC201 in vitro, we evaluated the expression of DRD5 in these archival tumor specimens by IHC analysis. In support of DRD5-low tumors being more responsive to ONC201, the 3 patients with PFS>5 month, used as a surrogate endpoint for clinical benefit in this patient population [26], uniformly had no detectable expression of DRD5 unlike those with PFS<5 months (Fig. 4A–B). Furthermore, patients with DRD5-low

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tumors exhibited a superior overall survival based on a DRD5 threshold defined to give the greatest separation in outcomes with 4/8 DRD5- and 0/7 DRD5+ patients at 15 months following initiation of ONC201 (Fig. 4C). This includes a recurrent GBM patient who had the H3F3A K27M somatic mutation in her tumor and has experienced a durable complete regression of her primary thalamic lesion and a 96% reduction in overall tumor burden (Fig. 4D–E).

Based on the notion that DRD2+DRD5- tumors may be optimally responsive to ONC201, we further explored TCGA for tumor types that contain this expression pattern (Fig. S5A). Several PCPG specimens exhibited relatively high expression of DRD2 without high expression of DRD5 that was also observed at the protein level (Fig. S5B). This tumor type is being evaluated in a dedicated cohort of an ongoing Phase II clinical trial with ONC201 (NCT03034200). Leiomyosarcoma was also found to exhibit this presumably favorable expression pattern (Fig. S5C). Leiomyosarcoma is a soft tissue sarcoma that often manifests in the uterus, which is interesting given that ONC201 has exhibited signs of clinical efficacy in endometrial cancer that is another type of uterine cancer being evaluated in clinical trials (NCT03099499; NCT03485729; NCT03394027).

Discussion

GPCRs are targeted by 30–50% of marketed drugs and are dysregulated in many types of human cancer, but have historically not been targeted in oncology outside of select neuroendocrine tumors [1]. Several studies support the notion that dopamine and other neurotransmitters play a key role in tumorigenesis and could serve as therapeutic targets [27]. Meta-analyses on cancer incidence in patients with Parkinson's disease and schizophrenia populations have found that dopamine pathway blockade is associated with lower levels of cancer [28, 29]. Our findings demonstrate that DRD2 is overexpressed in a number of tumor types and correlated with poor survival outcomes. In particular, DRD2 expression is dysregulated in gliomas that appear to be the tumor type that is most susceptible to DRD2 antagonism. These findings are consistent with other studies that have demonstrated DRD2 overexpression and the anti-cancer effects of DRD2 antagonism in various tumor types [2–4].

Dopamine receptors are classified as D1-like (DRD1 and DRD5) that associate with the G_s to activate adenylyl cyclase and D2-like (DRD2, DRD3, and DRD4 receptors) that couple with G_i to inhibit adenylyl cyclase activity [30]. A machine learning-based target identification platform [25] previously predicted that ONC201 directly antagonizes DRD2 and DRD3. β-arrestin and cAMP assays confirmed that ONC201 selectively antagonizes DRD2/3. Schild analyses and radioligand competition assays revealed DRD2 antagonism at concentrations consistent with ONC201 anticancer activity [25]. Induction of serum prolactin, a surrogate biomarker of DRD2 antagonism, was detected in ONC201-treated patients [10, 11]. Additionally, disrupting DRD2 expression in tumor cells modulated ONC201-mediated apoptosis and induction of integrated stress response [31].

In this study, we observed a correlation between DRD2 mRNA expression and ONC201 anti-cancer efficacy in select tumor types. We also found concordance between tumor type

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sensitivity to ONC201 and tumor type sensitivity to DRD2 genetic knockdown. Importantly, we found that selective targeting of DRD2 was superior to concurrent targeting of D1-like dopamine receptors. This suggests that the selectivity of ONC201, in addition to enabling its safety profile, may be critical for optimal anti-cancer activity that is more pronounced than many antipsychotics that antagonize dopamine receptors non-specifically.

While prior studies have evaluated roles for DRD2 [2–4] and DRD4 [32] in cancer, the role of DRD5 in cancer has not been well studied. One study showed that a DRD5 agonist suppresses pituitary, glioblastoma, colon and gastric tumor growth [33], while another revealed DRD5 upregulation in response to docetaxel in non-small cell lung cancer [34]. The results that we report implicate DRD5 as a negative regulator of DRD2 antagonism with utility that may be considered in distinct contexts. The first consideration is that innate tumor cell expression of DRD5 is inversely associated with DRD2-associated downstream signaling effects and therefore response to DRD2 antagonism. Accordingly, innate DRD5 expression was inversely correlated with ONC201 activity in vitro and in patients. A second consideration is that transient DRD5 activation in cancer cells leads to decreased DRD2-associated downstream signaling that will produce an anti-cancer response. This is consistent with the observation that tumor cells with stable acquired resistance to ONC201 possessed a Q366R mutation in the DRD5 gene that induced apoptosis upon expression in parental cells. These two distinct considerations may lead to the use of DRD5 as a predictive biomarker and as a therapeutic target in conjunction with DRD2 antagonism, respectively. Further to the former utility, a DRD2+DRD5- expression signature pointed to PCPG and uterine cancer as potential indications for ONC201 clinical studies. The expression signature could provide additional clinical opportunities to expand the utility of ONC201 in other gliomas with low DRD5 expression and guide the selection of other DRD5-low tumors beyond glioma for further evaluation with ONC201. Additionally, this predictive biomarker could help identify combinatorial therapies that can lower innate DRD5 expression and sensitize tumor cells to ONC201.

One limitation of this study is that while DRD2 is clearly overexpressed in cancer, the mechanism of DRD2 overexpression that can be detected at the mRNA level needs further study. The role of DRD2 and DRD5 homodimers versus heterodimers in tumor growth, spectrum of expression, and response to ONC201 should also explored. Finally, the use of intratumoral DRD5 expression to predict clinical outcomes in ONC201-treated high grade glioma patients needs to be validated prospectively in a larger number of patients and in other tumor types.

Together, our results posit DRD2 as a therapeutic target for oncology that can be selectively addressed by ONC201 and identify DRD5 as a direct negative regulator of DRD2 signaling and tumor cell response to its antagonism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Overexpression of dopamine receptor D2 (DRD2) is associated with a poor clinical prognosis in cancer patients. We demonstrate that DRD5, a dopamine receptor family member that opposes DRD2 signaling, is a negative regulator of tumor cell sensitivity to DRD2 antagonism. Small molecule ONC201 is the first selective antagonist of D2-like dopamine receptors for clinical oncology. We report a DRD2+DRD5- biomarker signature that was predictive of enhanced tumor cell sensitivity to ONC201 in preclinical models and is associated with improved outcomes in patients treated with ONC201 in a Phase II clinical trial for recurrent glioblastoma (NCT02525692). The predictive biomarker signature for ONC201 is under evaluation in ongoing glioma clinical trials and may be used to identify additional indications for ONC201, such pheochromocytoma that is now being investigated in a Phase II clinical trial (NCT03034200).

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Figure 1. DRD2 is selectively overexpressed in human cancer.

(**A**) Expression of DRD2 in human tumors and normal tissue in TCGA. (**B**) Z-score of DRD2 log(expression + 1) versus normalized survival values for different cancers in TCGA (P =.0348, Fisher's exact test). (**C**) Quantification and (**D**) exemplary images from DRD2 IHC analysis of tissue microarrays in GBM (n=35), neuroblastoma (n=25), medulloblastoma (n=20), endometrial cancer (n=32) and pheochromocytoma (n=30) with corresponding normal tissue. Dotted lines represents average expression for each tumor type. Quantification for normal tissue could not be performed due to low number of samples. Data is presented as DRD2-positive staining as a percentage of total tumor area.

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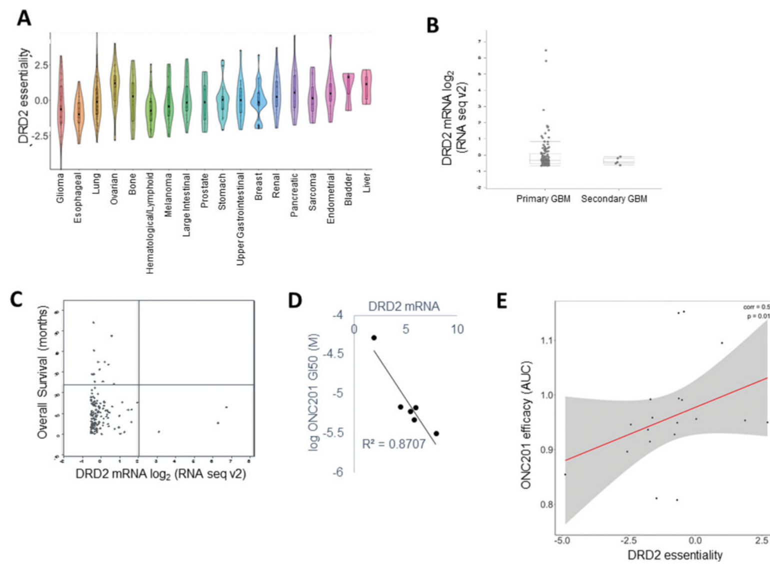

Figure 2. DRD2 dysregulation in human glioma and concordance with tumor response to DRD2 antagonism by ONC201.

(**A**) Distribution of DEMETER essentiality scores for DRD2 by primary tumor type. (**B**) Expression of dopamine receptors in GBM specimens by RNA-seq from TCGA by primary versus secondary GBM. (**C**) DRD2 expression in glioblastoma versus overall survival in patients. (**D**) ONC201 GI50 in NCI60 GBM cell lines versus DRD2 expression (48 h). (**E**) Scatter plot showing the correlation between brain cancer cell line-matched ONC201 AUC values and DRD2 DEMETER essentiality scores, with correlation and p-value calculated using Spearman correlation test.

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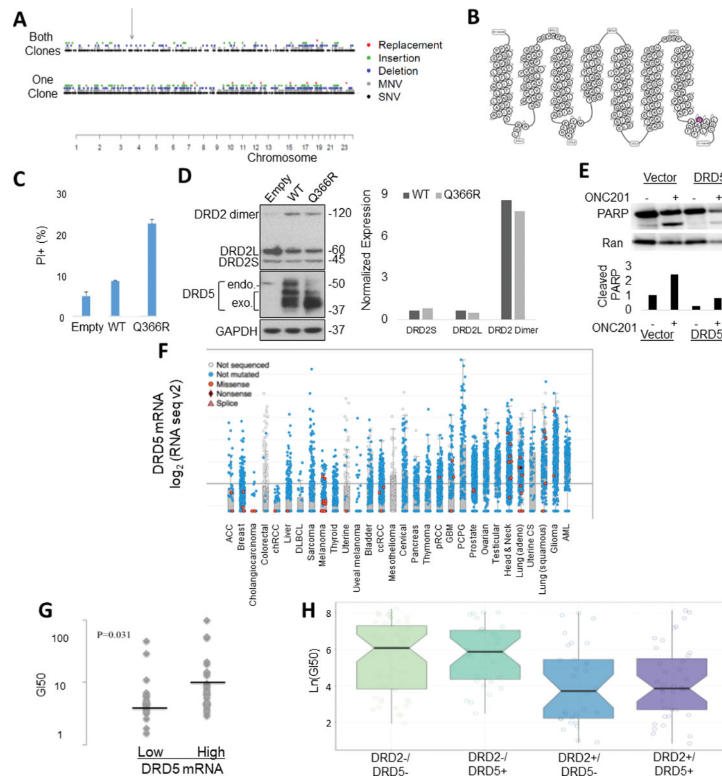

Figure 3. DRD5 is an inversely correlated predictive biomarker of tumor cell sensitivity to ONC201.

(**A**) DRD5 missense mutation identified by whole exome sequencing in RKO cells with acquired resistance to ONC201. Points are colored based on the type of genomic event. Overlapping mutation in DRD5 among resistant clones is annotated with an arrow. (**B**) Snake plot of Q366 amino acid location in DRD5 protein. (**C**) Propidium iodide staining and (**D**) Western blot analysis of RKO cancer cells following overexpression of wild-type or Q366R DRD5 constructs. Quantitation of DRD2 monomer and dimer expression normalized to GAPDH is shown on the right. (**E**) Western blot analysis of PARP cleavage in HCT116 cancer cells treated with ONC201 with overexpression of wild-type or Q366R DRD5. Quantitation of cleaved PARP normalized to total PARP and Ran is shown at the bottom. (**F**) Expression of DRD5 in human tumor samples in TCGA. (**G**) ONC201 GI50 of NCI60 cells categorized by DRD5 mRNA expression using z-score (48 h). (**H**) ONC201 efficacy distributions for DRD2+/DRD5- and DRD2-/DRD5- samples in the GDSC panel (P = .0037, KS Test, 72 h). Cutoffs for DRD2 expression were z-scores of 1 and −1. Cutoffs for DRD5 were z-scores of 0.5 and −0.5.

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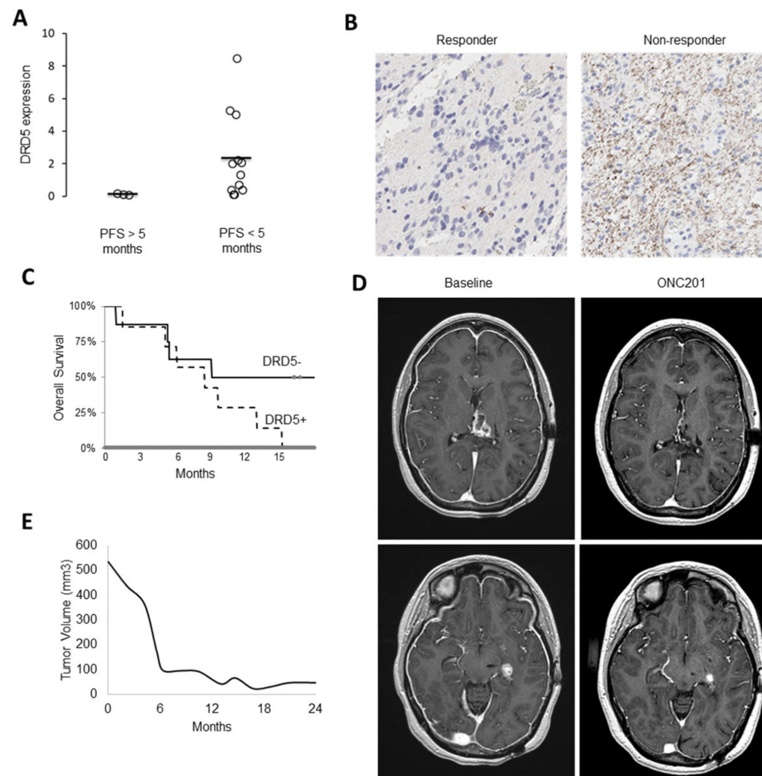

Figure 4. Intratumoral DRD5 expression is associated with clinical outcomes of ONC201-treated adult recurrent glioblastoma patients.

(**A**) DRD5 expression by IHC analysis in archival tumor samples categorized by PFS>5 months (n=3) or PFS<5 months (n=12) of ONC201-treated recurrent GBM patients (625mg q3w PO). Data is presented as DRD5-positive staining as a percentage of total tumor area. (**B**) Exemplary DRD5 expression by IHC analysis in archival tumor specimens from ONC201-treated recurrent GBM patients who experience an objective response or progressive disease. (**C**) Overall survival of ONC201-treated recurrent GBM patients who had archival tumor specimens that were DRD5- versus DRD5+ by IHC analysis. Gray circles indicate a censoring event for followup. (**D**) Thalamic and (**E**) parietal lobe GBM lesions before and 17 months after 625mg q3w ONC201. (**F**) Overall tumor burden over time in responding recurrent GBM patient treated with ONC201.