Role of Dopamine Receptors in the Anticancer Activity of ONC201

Christina Leah B. Kline*, Marie D. Ralff*, Amriti R. Lulla*, Jessica M. Wagner*, Phillip H. Abbosh*, David T. Dicker*, Joshua E. Allen† and Wafik S. El-Deiry*

*Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Molecular Therapeutics Program, Department of Hematology/Oncology, Fox Chase Cancer Center, Philadelphia, PA; †Oncoceutics, Inc., Philadelphia, PA

Abstract

ONC201/TIC10 is a first-in-class small molecule inducer of TRAIL that causes early activation of the integrated stress response. Its promising safety profile and broad-spectrum efficacy in vitro have been confirmed in Phase I/II trials in several advanced malignancies. Binding and reporter assays have shown that ONC201 is a selective antagonist of the dopamine D2-like receptors, specifically, DRD2 and DRD3. We hypothesized that ONC201’s interaction with DRD2 plays a role in ONC201’s anticancer effects. Using cBioportal and quantitative reverse-transcription polymerase chain reaction analyses, we confirmed that DRD2 is expressed in different cancer cell types in a cell type–specific manner. On the other hand, DRD3 was generally not detectable. Overexpressing DRD2 in cells with low DRD2 levels increased ONC201-induced PARP cleavage, which was preceded and correlated with an increase in ONC201-induced CHOP mRNA expression. On the other hand, knocking out DRD2 using CRISPR/Cas9 in three cancer cell lines was not sufficient to abrogate ONC201’s anticancer effects. Although ONC201’s anticancer activity was not dependent on DRD2 expression in the cancer cell types tested, we assessed the cytotoxic potential of DRD2 blockade. Transient DRD2 knockdown in HCT116 cells activated the integrated stress response and reduced cell number. Pharmacological antagonism of DRD2 significantly reduced cell viability. Thus, we demonstrate in this study that disrupting dopamine receptor expression and activity can have cytotoxic effects that may at least be in part due to the activation of the integrated stress response. On the other hand, ONC201’s anticancer activity goes beyond its ability to antagonize DRD2, potentially due to ONC201’s ability to activate other pathways that are independent of DRD2. Nevertheless, blocking the dopamine D1-like receptor DRD5 via siRNA or the use of a pharmacological antagonist promoted ONC201-induced anticancer activity.

Neoplasia (2018) 20, 80–91
Introduction

Dopamine receptors respond to the neurotransmitter dopamine. These receptors are G-protein coupled receptors (GPCRs) and can be divided into two major groups: D1-like and D2-like. The D1-type receptors (DRD1 and DRD5) generally associate with the G\(_{\text{i/o}}\) subunit and, consequently, activate adenylyl cyclase. By contrast, D2-like receptors (DRD2, DRD3, and DRD4 receptors) usually couple with G\(_{\text{i/o}}\) subunit and inhibit adenylyl cyclase activity [1]. Dopamine receptors have been studied mostly in the context of neurobiology. Their role in cancer remains unclear and appears to be highly tumor type specific. In a number of cancer types, D2-like receptor activation inhibits cancer cell proliferation [2] or induces apoptosis. However, in different contexts, D2-like receptor antagonism has been shown to have anticancer effects [3–6]. The mechanism of this efficacy involves, at least in part, the activation of the cAMP/PKA pathway [3].

Computational methods have suggested that the first-in-class small molecule ONC201 may be a selective antagonist of the dopamine receptors of the D2-like class. In vitro experiments have confirmed that ONC201 is a direct competitive antagonist of dopamine receptors DRD2 and DRD3, with a \(K_i\) of 3 \(\mu\)M [7,8]. Patients receiving ONC201 in Phase I/Phase II clinical trials have shown a two-fold induction of serum prolactin, a marker for DRD2 receptor engagement [9].

ONC201 (originally known as TIC10) is a promising first-in-class small molecule that we screened and identified by its ability to induce expression of the death ligand Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) in a p53-independent manner [10]. ONC201 treatment results in dual inactivation of Akt and ERK, Foxo3a activation, and TRAIL induction. More recently, we and others reported that ONC201 induces an early activation of the integrated stress response (ISR), as indicated by the consequent upregulation of ATF4 and one of ATF4’s transcriptional target, CHOP. ONC201’s ability to provoke the ISR is dependent on two eIF2\(\alpha\) kinases, HRI and PKR [11]. In the study reported here, we explored the possibility that the putative binding of ONC201 with DRD2 and DRD3 may contribute to ISR activation and anticancer activity.

Materials and Methods

Cell Culture

The human tumor cell lines were acquired from the American Type Culture Collection or the Fox Chase Cancer Center Cell Culture Facility. Cell line authentication was performed using short tandem repeat methodology [12]. All cell lines used in this study were confirmed to be free of mycoplasma contamination. The human lung, colorectal, liver, and prostate cancer cell lines were cultured in RPMI or McCoy’s 5A medium. Human breast, glioblastoma, and neuroblastoma cell lines were maintained in Dulbecco’s minimum essential medium. The ONC201/TIC10-resistant derivative of RKO colorectal cancer cells was previously described [11]. Nontumorigenic cells were cultured in Eagle’s minimum essential medium. All basal media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

RNA was isolated using RNeasy kit (Qiagen) or Quick-RNA Miniprep kit (Zymo Research) according to manufacturers’ instructions. RNA was quantitated using a Nanodrop spectrophotometer. cDNA was synthesized using a SuperScript II RT kit, while real-time PCR was performed using a Quantitect SYBR Green PCR mix. Relative amounts of target mRNA were quantitated using the 2\(^{-\Delta\Delta CT}\) method using GAPDH as internal control. At least three technical replicates per biological replicate were analyzed.

Immunoblotting

Cultures were washed twice with PBS, and the cells were lysed into lysis buffer (50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 0.5% NP40, 10% glycerol, supplemented with 0.0001% Tween 20, 0.1 mM PMSF, 0.1 mM NaVO\(_4\), 0.5 mM NaF, 5 \(\mu\)g/ml leupeptin, 0.1 mM DTT). The proteins were quantified with the Bio-Rad protein assay and loaded equally onto 4% to 12% NuPage SDS-polyacrylamide gels (Inviroteg). Proteins were electrophoretically transferred to PVDF membranes. After blocking with 5% BSA or 5% milk, membranes were incubated with primary antibody overnight. Subsequently, incubation with appropriate secondary antibodies labeled with horseradish peroxidase was performed. Signal was visualized using chemiluminescence detection. The primary antibodies used in this study were: antibodies against PKA substrate (cat. # 9621S), ATF4 (cat. # 11815S), CHOP (cat. # 2895S), phospho-ERK1/2 (cat. # 4377S), ERK (cat. # 9102S), and PARP (cat. # 9542S) (Cell Signaling); against Ran (cat. # 610341) (BD Biosciences); and against β-actin (Sigma). Secondary antibodies were acquired from Pierce (cat. # 31430 and 31,460).

Plasmid Overexpression

Plasmid DNA constructs were acquired from Addgene and amplified according to Addgene recommendations. Plasmid DNA was isolated using QIAprep Spin midiprep kit (Qiagen) according to manufacturer’s instructions. A total of 0.5 to 1 \(\mu\)g DNA was transfected into cells using Lipofectamine 2000. Vehicle or ONC201 was added 24 hours later, when indicated. GFP-DRD2 expression was verified by GFP-fluorescence imaging with a Nikon Eclipse-Ti-U inverted microscope.

Knockout of DRD2 and DRD3 Expression Using CRISPR

pLCV2E (Addgene #78852) was generated by PCR amplification of the C-terminus of the ORF containing eSpCas9(9.1) (Addgene #71814). PCR primers contained EcoRV and BamHI restriction sites to facilitate cloning into plasmid pLCV2 (Addgene #52961). This fragment contains the three point mutations which enhance specificity of codon-optimized SpCas9 and maintains the ORF of the polypeptide encoding puromycin acetyltransferase. The plasmid was sequence verified between restriction sites.

Single-guide RNAs (sgRNAs) were chosen using sgRNA Designer. For DRD2, RefSeq NM_016574.3 was used. On-target scores >0.6 were chosen such that several high-scoring sgRNAs could be screened using the same amplicon in a Surveyor or TIDE assay. Off-target scores were largely neglected since the eSpCas9 enzyme has low off-target rates. All sgRNAs were targeted to sequences encoding the 7-transmembrane domain. All chosen sgRNAs had a native 5’ G nucleotide which enhances expression from a U6 promoter and avoids potential mismatches which may limit on-target...
efficiency. Guides were cloned into the BsmBI site of pLCV2E, and sequence was confirmed. Sequences for single-guide RNAs and amplicon primers are as follows:

Guide RNAs for DRD2 deletion:

| Guide   | Sequence                        |
|---------|---------------------------------|
| Guide 1 | GATGGAGAGGTAGACCGAGCA           |
| Guide 3 | GATCTTGGATGAGGACGCCA            |

Guide RNAs for DRD3 deletion

| Guide   | Sequence                        |
|---------|---------------------------------|
| Guide 4 | GCACCGGAGCAGACTCGTGT            |
| Guide 5 | GATAAACGCAAGCAAGAAG             |
| Guide 6 | GAAGCCATGACTGTATCTGGT           |

Primer sequences for sequence verification

Hs DRD2 forward: GTGACATCTAAAGGGCCAGCC
Hs DRD2 reverse: GTGAGCCTTAAAGGGCCAGGC
Hs DRD3 forward: ACGAATCTCTTCTTTCGTGTC
Hs DRD3 reverse: AAGCTTTGGATCTTGCGCA

Viability and Apoptosis Assays

Viability was assayed using the CellTiter-Glo (CTG) assay according to the manufacturer’s instructions (Promega). A total of $1 \times 10^5$ cells were seeded in 96-well black plates and incubated for 24 hours prior to addition of agents. To assay viability by a different method, trypan blue dye exclusion assays were performed. The cells were cultured in 24-well plates, treated with vehicle or ONC201, harvested, and stained with trypan blue. Viable cells were counted using a Cellometer cell counter.

Sub-G1 analyses were performed to quantify apoptosis. After treatment, floating and adherent cells were fixed in 95% ethanol and centrifuged. The supernatant was used to infect HCT116, HT29, or SKNSH cells with 8 μg/ml Polybrene. After 2 days, cells were trypsinized, plated at 10% confluency, and then treated with 1 μg/ml puromycin. Cells were passaged in selective medium at least two times prior to collection for DNA isolation.

Transfection experiments were performed using Accuprime PFX (Life Technologies) using touchdown PCR. The starting amplification temperature was 65°C and was decreased by 0.5°C/cycle for 10 cycles. Amplification at 60°C was continued for 20 additional cycles. One microliter of PCR product was diluted and sequenced (GeneWiz). Unmodified DNA was inputted into TIDE [13] (https://tide-calculator.nki.nl/) to determine indel rates.

Transient Knockdown Using siRNA

Cells were plated in medium with 10% FBS but without antibiotic and incubated overnight. siRNA (a pool of three target-specific siRNAs) (Santa Cruz Biotechnology) (40 nM final concentration) was transfected into cells using 9 μl Lipofectamine RNAiMax. After 24 hours, vehicle or ONC201 was added. In the case of double knockdowns, the final concentration of each siRNA was 20 nM.

Stable Knockdown Using shRNA

Five different lentiviral plasmid shRNA constructs for DRD2 (Origene) were amplified, and DNA was isolated using plasmid mini kit (Qiagen) according to manufacturer’s instructions. Lentiviral particles were generated by transfecting HEK293T cells with shDRD2 DNA, packaging plasmids pCMV-VSV-G, and pCMV delta R8.2 in 2:1:1 ration. After 72 hours, lentiviral particles were collected, diluted with equal volume of media, and added to cultures of HCT116 cells. Stable transfectants were selected with 1 μg/ml puromycin. Knockdown efficiency was assessed by qRT-PCR analysis for DRD2 mRNA expression.

Statistics

Data are presented as means ± SEM. To assess the statistical significance of the differences, unpaired Student’s t test with Holm-Sidak correction for multiple comparisons (maximum of three comparisons were made) was performed with $P < .05$ deemed as statistically significant. Measurements from three biological replicates per treatment group were compared. Unless otherwise noted in the figure legend, comparisons were made against the vehicle control.

Results

Expression and Activity of D2-Like Receptors in Colorectal Cancer Cells

Given that dopamine receptors are mostly studied in the context of neurobiology, we explored the expression pattern of the receptors across different cancer cell types. To begin to interrogate the impact of this molecular interaction on ONC201’s anticancer activity, we utilized the cBioPortal for Cancer Genomics resource to assess DRD2 mRNA expression across multiple cancer types. We found that DRD2 expression is highly variable across different cancers (Figure 1) [14,15]. We evaluated the expression of the different dopamine receptors in seven cancer cell lines, including the ONC201/TIC10-resistant derivative of RKO colorectal cancer cells, and two nontumorigenic lines (Table 1). The D1-like receptors (DRD1 and DRD5) were more highly expressed in contrast, expression of DRD3 was not detectable in most cancers tested (Figure S1) [14,15]. We evaluated the expression of the different dopamine receptors in seven cancer cell lines, including the ONC201/TIC10-resistant derivative of RKO colorectal cancer cells, and two nontumorigenic lines (Table 1). The D1-like receptors (DRD1 and DRD5) were more highly expressed in the tumor cell lines than in the normal lines, as indicated by their lower delta Ct values. By contrast, the expression of the D2-like receptors was highly cell-type specific. A delta Ct value higher than 19.93 indicates that the receptor mRNA level is >10,000-fold lower than that of the housekeeping gene GAPDH. Similar to the results from using cBioportal, DRD3 expression was not appreciable in any of the nine cell lines tested.

Engagement of the D2-like receptors can promote cell proliferation [16,17]. Thus, to confirm that the expressed dopamine receptors, as 0.25% crystal violet-methanol solution for 30 minutes. After washing stain off with water, plates were allowed to dry and colonies were counted manually.
Figure 1. D2-like receptors are expressed and functional in colorectal cancer cells. (A) Assessment of DRD2 mRNA expression in different cancer tissue samples using cBioportal. (B) Viability assessment after 72 hours of treatment with dopamine or sumanirole, as indicated in legend. Western blot analyses of PKA substrate phosphorylation in (C) HCT116 cells treated with 500 μM phosphodiesterase inhibitor IBMX and 10 μM adenylyl cyclase activator forskolin (F), or treated with 10 μM ONC201, in the presence or absence of 1 μM protein kinase A inhibitor peptide 6-22 (PKI); (D) Hep3B cells treated with different doses of ONC201 for 24 hours; (E) HCT116, RKO, and ONC201-resistant RKO cells (RKO1) treated with 10 μM (HCT116) or 5 μM (RKO and RKO1) ONC201 for indicated times.
determined by qPCR, are functional in the cancer cell types to be used in the study, we assayed the impact of dopamine and the selective D2-like receptor antagonist sumanirrole on cell viability of HCT116 and RKO colorectal cancer cells. Both dopamine and sumanirrole induced an increase in cell viability (Figure 1B).

The binding of ligands to dopamine receptors can perturb cyclic AMP (cAMP) levels as a result of alteration in adenylyl cyclase activity [1]. D2-like receptor antagonists can induce an increase in cAMP [18,19] and activate cAMP-dependent protein kinase (PKA) [20,21]. Thus, we assessed ONC201’s effects on PKA activity. As a positive control for activation of PKA, HCT116 cells were treated with forskolin, an adenylyl cyclase activator, in the presence of the control for activation of PKA, HCT116 cells were treated with forskolin, an adenylyl cyclase activator, in the presence of the phosphodiesterase inhibitor 3-isobutyl 1-methylxanthine (IBMX) (Figure 1C). ONC201 treatment induced PKA substrate phosphorylation with differential kinetics in cancer and normal cell lines (Figure 1, D-E).

**Cell-Type Specific Role of DRD2 in ONC201’s Anticancer Effect**

To assess whether the putative binding of ONC201 with DRD2 contributes to ONC201-induced ISR activation and ONC201’s anticancer effects, we modulated the expression of DRD2 and performed assays for ATF4 and CHOP expression and PARP cleavage.

We overexpressed DRD2 in two cancer cell lines that did not express appreciable levels of DRD2 (Table 1)—the colorectal cancer cell line RKO and the hepatocellular carcinoma cell line Hep3B—to assess the impact of increased receptor levels on sensitivity to ONC201. Fluorescence imaging confirmed DRD2 expression, especially in the cell membrane (Figure 2A). Overexpressing the receptor increased the extent of PARP cleavage resulting from ONC201 treatment (Figure 2B).

| Cell type | Cell Line | DRD1 | DRD2 | DRD3 | DRD4 | DRD5 |
|-----------|-----------|------|------|------|------|------|
| Normal    | CCD CON   | 15.4 | 20.8 | ND   | 20.5 | 17.8 |
|           | IMR90     | 14.6 | 20.6 | 27.03| 18   | 19.7 |
|           | HCT116    | 10.4 | 17.8 | ND   | 21.5 | 12.1 |
|           | HT29      | 14.4 | 16.9 | ND   | 20.8 | 13.8 |
|           | RKO       | 11.9 | ND   | 22.5 | ND   | 14.3 |
|           | RKO1r     | 11.1 | 21.0 | 21.8 | ND   | 10   |
| Colon     | U251      | 10.8 | 6.0  | ND   | 22.1 | 10.3 |
|           | SKNSH     | 11.7 | 18.7 | ND   | 17.5 | 18   |
|           | Hep3B     | 13.7 | 23.2 | ND   | 29.6 | 15.1 |

Table 1. The Expression of the Different Dopamine Receptors Is Variable Across Different Cell Types (ΔCt vs GAPDH Are Shown)

Delta Cts higher than 19.93 indicate that they are 10,000-fold less abundant than GAPDH. Data highlighted in green indicate appreciable receptor mRNA expression in indicated cell lines. ND, not detected even if Gt value of GAPDH is ±17.

The increase in anticancer activity of ONC201 in DRD2-overexpressing RKO cells was correlated with an upregulation in CHOP mRNA expression (Figure 2C). Although DRD2 overexpression increased ONC201-induced PARP cleavage in wild-type RKO cells, DRD2 overexpression was not sufficient to increase PARP cleavage in ONC201/TIC10-resistant RKO cells (Figure S2, A-B).

We knocked out DRD2 protein expression via CRISPR-Cas9-mediated gene disruption. DNA sequencing confirmed a high percentage of indel mutations in cells that were selected after transfecting with guide RNA #3 (Figure S2C). Knocking out DRD2 in HCT116 and HT29 colorectal cancer cells did not significantly abrogate ONC201’s effects on cell number. In SKNSH neuroblastoma cells, the knockout of DRD2 increased the effect of ONC201 on cell count, albeit its effect was not statistically significant (p = .08 when wt SKNSH plus ONC201 was compared to Guide 3 [G3] SKNSH plus ONC201) (Figure 2D). The D2-receptor antagonist L-741,626 decreases cell viability, like ONC201. Knocking out DRD2 partially abrogated the cytotoxic effect of L-741,626 but not of ONC201 (Figure 2E). Clonogenic assays confirmed that DRD2 knockout did not significantly affect ONC201’s anticancer activity in HCT116 and HT29 cells (Figure S2D).

We explored whether the absence of a significant impact of DRD2 knockout on ONC201-induced qRT-PCR analyses can be explained by a compensatory overexpression of other dopamine receptors. qRT-PCR analyses showed that there was no increase in expression of the other receptors. Instead, expression of DRD1 was also significantly reduced with DRD2 gene deletion in HCT116 cells (Figure 2F). DRD4 was not detected in both HCT116 and HT29 cells, and DRD2 and DRD3 mRNAs were also not detected in G3 HT29 cells.

We have previously shown that breast cancer cells respond to ONC201 [22]. Thus, similar CRISPR/Cas-9 deletion experiments were performed with two breast cancer cell lines, MDA-MB231 and SUM149PT. Moreover, given that ONC201 has been shown to bind to another D2-like receptor, DRD3 [8], we assessed the impact of DRD3 knockout on ONC201 anticancer effects. Similar to what we have observed with DRD2, knockout of DRD3 or DRD5 was not sufficient to abrogate ONC201’s cytotoxicity in the two breast cancer cell lines (Figure S2, E-F).

**Consequences of Transient Knockdown of DRD2 in Colorectal Cancer Cells**

Although ONC201’s anticancer activity cannot be explained entirely by ONC201’s interaction with DRD2, we explored the contribution and potential of DRD2 antagonism as a therapeutic strategy, particularly in colorectal cancer cells. As has been previously shown [11], ONC201 decreases cell count. Transiently reducing DRD2 expression levels using siRNA was also sufficient to cause a decrease in cell number. Nevertheless,
Figure 2. ONC201’s anticancer effect is not dependent on DRD2 in a subset of cancer cell types. (A) Fluorescence imaging to verify expression of GFP-DRD2 in the membrane of transfected cells. (B) Western blot analyses of PARP cleavage in RKO cells transfected with GFP-DRD2 construct for 24 hours and subsequently treated with 5 μM ONC201 for 72 hours. Blots are representative of two experiments. (C) qRT-PCR analyses of CHOP and DR5 mRNA expression in RKO cells transfected with GFP-DRD2 and subsequently treated with 5 μM ONC201 for 24 hours. (D) Cell count after 48-hour treatment with ONC201. (E) Viability assessment after 72 hours of treatment with ONC201 or L-741,626. *P < .05 versus viability of wild-type cells similarly treated. (F) qRT-PCR analyses of basal dopamine receptor expression of wild-type and DRD2 CRISPR/Cas9 knockout cells (transfected with guide 3 RNA). Data are means ± SE from at least two biological replicates.
ONC201 had a significantly greater effect on reducing cell count than DRD2 knockdown (Figure 3A). DRD2 knockdown was not sufficient to cause apoptosis as assessed by PARP cleavage. However, DRD2 knockdown exacerbated ONC201-induced PARP cleavage (Figure 3B). This increase in ONC201-induced apoptosis as a result of DRD2 knockdown was preceded by an increase in ATF4 protein expression at 12 hours and CHOP protein expression at 24 hours (Figure 3C).

As an alternative strategy to knock down DRD2 expression, we stably transfected HCT116 cells with DRD2 shRNA. Quantitative PCR results confirmed DRD2 knockdown in the transfectants. Moreover, one of the two clones (shRNA #43) showed a downregulation of DRD1 (Figure 3D). Similar to ONC201’s effects, knocking down DRD2 resulted in PKA activation (Figure 3E). In contrast to the effect of transient DRD2 knockdown on cell number (Figure 3A), the growth rate of HCT116 cells was not consistently downregulated when DRD2 expression levels were stably reduced (Figure 3F).

These results suggest that transient knockdown of DRD2 can reduce colorectal cancer cell number, at least in part, as a consequence of the activation of the integrated stress response.

Figure 2. (continued.)
Effects of Selective D2-Like Receptor Antagonists in Colorectal Cancer Cells

Given that transient knockdown of DRD2 decreased colorectal cancer cell number, we assessed the cytotoxic effects of other D2-selective antagonists. Although all the antagonists tested were specific for the D2-like receptor class, they had different preferential selectivity for DRD2 or DRD3. Remoxipride had similar micromolar affinities for the D2-like receptors as ONC201 (Table 2). Out of the D2-antagonists tested, L-741,626 and PG01037 also [23] significantly decreased cell viability. Their respective EC50s were in the micromolar range (Figure 4A). L-741,626 did not generally induce apoptosis (except in RKO cells) (Figure 4, B-C) or activate the ISR, at least under the conditions tested in this study (Figure 4D). PG01037 also did not induce PARP cleavage (Figure S3). ONC201 has been

Figure 3. Transient knockdown of DRD2 in colorectal cancer cells can activate the ISR and reduce cell number. (A) Viable cell count. (B) Western blot analyses of PARP cleavage and CHOP expression. (C) Western blot analyses of ATF4 and CHOP protein expression in cells after DRD2 siRNA-mediated knockdown for 24 hours and subsequent treatment with 10 μM ONC201 for indicated times. *P < .05 versus vehicle-treated scramble siRNA-transfected; P < .05 versus vehicle-treated DRD2 siRNA-transfected. (D) qRT-PCR analyses of DRD1 and DRD2 mRNA expression were performed to verify knockdown and monitor for potential compensatory overexpression of DRD1 receptor. Data are means ± SE from three biological replicates. P < .05 versus viability of control shRNA cells similarly treated. (E) Western blot analyses for PKA substrate phosphorylation in stably transfected control and DRD2 shRNA cells treated with 10 μM ONC201 for 24 hours. (F) Cell proliferation rate assessment of control and DRD2 shRNA-transfected cells was performed by enumerating cell number after indicated times of cell culture. Data are means ± SE from three biological replicates. P < .05 versus viability of control shRNA cells similarly treated.
shown to be cancer selective [24]. By contrast, L-741,626 had significant cytotoxic activity against normal cells (Figure 4E).

Effects of DRD5 Downregulation on Sensitivity to ONC201

DNA sequencing of ONC201-resistant RKO cells uncovered a missense mutation in dopamine receptor DRD5 [25]. Given that all of the cancer cell types tested in this study express DRD5 (Table 1), we transiently knocked down DRD5 and assessed whether ONC201-induced anticancer activity would be increased. Knocking down DRD5, but not the other D1-like receptor DRD1, increased ONC201-induced PARP cleavage (Figure 5A). Alternatively, we assessed whether treating cancer cells with a selective D1/D5

| Affinity of the Different D2-Like Receptor Antagonists Tested in This Study |
|-----------------|-----------------|-----------------|-----------------|
| $K_i$ (nM)      | ONC201          | L-741,626       | PG01037         | Remoxipride     |
| D2-like receptors | [33]            | [23,34]         | [33]            | [36]            |
| DRD2            | 3000 2.4 0.3 $^a$ | 93.3 30 $^a$    |                 |                 |
| DRD3            | 3000 100        | 0.7 1600        |                 |                 |
| DRD4            | 220             | 375 2800        |                 |                 |

$^a$ Radioligand independent [36].

Figure 3. (continued.)
antagonist, SCH39166 hydrobromide [26], can increase ONC201’s effects on cell viability. The two colorectal cell lines HCT116 and RKO were relatively resistant to up to 50 μM SCH39166 (Figure S4). On the other hand, the neuroblastoma cell line SKNSH and the glioblastoma cell line U251 were sensitive to 50 μM SCH39166. Nevertheless, antagonizing D1/D5 with SCH39166 significantly increased susceptibility to ONC201 (Figure 5B).

Discussion
The ability of ONC201 to bind to the D2-like receptor proteins, as has been observed in heterologous systems, provides an opportunity to explore the therapeutic potential of targeting these GPCR proteins in the context of cancer. There have been a number of papers that show the potential anticancer activity of D2-like receptor antagonists. In pancreatic cells [3] and glioblastoma [5], DRD2 knockdown is...
sufficient to significantly reduce cell viability. Single-agent treatment with D2-like receptor antagonists has been shown to be antitumorigenic against cervical and endometrial cancer, melanoma cells [27,28], and breast cancer stem cells [4]. A limitation of these latter studies, however, is the lack of evidence that the effects of the antagonists were due to the D2-like receptors and not due to off-target effects. Moreover, a number of the antagonists that have been tested (e.g., haloperidol, thioridazine) have other targets aside from the D2-like receptors. By contrast, ONC201, L-741,626, PG01037, and remoxipride have not been shown to have other direct-binding targets aside from the D2-like receptors. Thus, these small molecules are appropriate tools to test the anticancer effects of D2-like receptor antagonism. We demonstrated in this study that most of the D2-receptor antagonists tested had cytotoxic effects. On the other hand, only ONC201 had significant proapoptotic effects. We explored the effect of DRD2 knockdown on the viability of colorectal cancers, in particular. We demonstrated that transient knockdown of DRD2 can have antiproliferative activity in colon cancer cells is helpful not only in understanding the effects of DRD2-targeted drugs but also in thinking of new ways of using these important neurobiological therapeutics.

Acknowledgements
Oncoceutics provided ONC201. This work was presented in part at the 2016 and 2017 American Association for Cancer Research meetings. This work was supported by grants from the National Institutes of Health (R01 CA173453) and the American Cancer Society to W. S. E.-D. W. S. E.-D. is an American Cancer Society Research Professor.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2017.10.002.

References
[1] Beaulieu JM, Espinosa S, and Gainetdinov RR (2015). Dopamine receptors — IUPHAR Review 13. Br J Pharmacol 172, 1–23.
[2] Roy S, Lu K, Nayak MK, Bhuinya A, Ghosh T, Kundu S, Ghosh S, Baral R, Dagupta PS, and Basu S (2017). Activation of D2 dopamine receptors in CD133+ve cancer stem cells in non-small cell lung carcinoma inhibits proliferation, clonogenic ability, and invasiveness of these cells. J Biol Chem 292, 435–445.
[3] Jandaghi P, Najafabadi HS, Bauer AS, Papadakis AI, Fassan M, Hall A, Monast A, von Knebel Doeberitz M, Neoptoloemos JP, and Costello E, et al (2016). Expression of DRD2 is increased in human pancreatic ductal adenocarcinoma and inhibitors slow tumor growth in mice. Gastroenterology 151, 1218–1231.

Figure 5. Downregulation of the D1-like receptor DRD5 increases sensitivity to ONC201. (A) Western blot analyses of PARP cleavage in cells after DRD1 or DRD5 siRNA-mediated knockdown for 24 hours and subsequent treatment with 10 μM ONC201 for 72 hours. (B) Viability assessment of indicated cell lines treated with vehicle, 5 μM ONC201, 50 μM SCH39166, or ONC201-SCH39166 combination for 72 hours.*P < .05 versus vehicle-treated cells; **P < .01 versus vehicle-treated cells; ***P < .001 versus vehicle-treated cells.
Neoplasia  Vol. 20, No. 1, 2018

Involvement of DRD2 and DRD5 in Anticancer Activity of ONC201  Kline et al.  91

[4] Sachlos E, Risueno RM, Laronde S, Shapirovalova Z, Lee JH, Russell J, Malig M, McNicol JD, Fiebig-Cornyn A, and Graham M, et al (2012). Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. Cell 149, 1284–1297.

[5] Li J, Zhu S, Kozono D, Ngi K, Futalan D, Shen Y, Akers JC, Steed T, KUshwaha D, and Schlabach M, et al (2014). Genome-wide shRNA screen revealed integrated mitogenic signaling between dopamine receptor D2 (DRD2) and epidermal growth factor receptor (EGFR) in glioblastoma. Oncotarget 5, 882–893.

[6] Bau S and Dasgupta PS (2000). Role of dopamine in malignant tumor growth. Endocrine 12, 237–241.

[7] Allen JE, Kline CL, Prabhu VV, Wagner J, Ishizawa J, Madhukar N, Le A, Baumeister M, Zhou L, and Lulla A, et al (2016). Discovery and clinical introduction of first-in-class imipridone ONC201. Oncotarget 7, 74380–74392.

[8] Madhukar NS, Khade PK, Huang L, Gayvert K, Galletti G, Aksoy BA, Stogniew M, Allen JE, Giannalakou P, and Elemento O (2017). A new big-data paradigm for target identification and drug discovery. bioRxiv; 2017.

[9] Stein MN, Bertino JR, Kaufman HL, Mayer T, Moss R, Silk A, Chan N, Al-Ahmadie H, and Scott J, et al (2017). First-in-human clinical trial of oral ONC201 in patients with refractory solid tumors. Clin Cancer Res [https://doi.org/10.1158/1078-0432.CCR-16-2658].

[10] Allen JE, Krigsveld G, Mayes PA, Patel I, Dicker DT, Patel AS, Dolloff NG, Mosaris E, Scata KA, and Wang W, et al (2013). Dual inactivation of Akt and ERK by TIC10 signals Fos/3a nuclear translocation, TRAIL gene induction and potent antitumor effects. Sci Transl Med 5, 1–13.

[11] Kline CL, Van den Heuvel AP, Allen JE, Prabhu VV, Dicker DT, and El-Deiry WS (2016). ONC201 kills solid tumor cells by triggering an integrated stress response dependent on ATF4 activation by specific eIF2alpha kinases. Sci Signal 9, r18.

[12] ASN-0002-2011 AA. Authentication of human cell lines: standardization of STR profiling; 2011.

[13] Brinkman EK, Chen T, Amendola M, and van Steensel B (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res 42, e168.

[14] Cerami E, Gazouz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Bryne CJ, Heuer ML, and Larsson E, et al (2012). The eBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2, 401–404.

[15] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, and Larsson E, et al (2013). Integrative analysis of complex cancer genomics and clinical profiles using the eBioPortal. Sci Signal 6, p11.

[16] Luo Y, Koklenen GC, Wang X, Neve KA, and Roth GS (1998). D2 dopamine receptors stimulate mitogenesis through pertussis toxin-sensitive G proteins and Ras-involved ERK and SAP/JNK pathways in rat C6-D21 glioma cells. J Neurochem 71, 980–990.

[17] Taylor M, Grundt P, Griffin SA, Newman AH, and Roth GS (2010). Dopamine D3 receptor selective ligands with varying intrinsic efficacies at adenylyl cyclase inhibition and mitogenic signaling pathways. Synapse 65, 251–266.

[18] Kaneko M, Sato K, Horikoshi R, Yagimana M, Yagimuna N, Shiragata M, and Kumasiro H (1992). Effect of haloperidol on cyclic AMP and inositol trisphosphate in rat striatum in vivo. Prostaglandins Leukot Essent Fatty Acids 46, 53–57.

[19] Maari B, Salikhov M, Ghisi V, Beaulieu JM, Gainerdinov RR, and Caron MG (2008). Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. Proc Natl Acad Sci U S A 105, 13656–13661.

[20] Dwivedi Y, Rizavi HS, and Pandey GN (2002). Differential effects of haloperidol and clozapine on [3H]cAMP binding, protein kinase A (PKA) activity, and mRNA and protein expression of selective regulatory and catalytic subunit isoforms of PKA in rat brain. J Pharmacol Exp Ther 301, 197–209.

[21] Sprunger JU and Nikolaev VO (2013). Biophysical techniques for detection of cAMP and cGMP in living cells. Int J Mol Sci 14, 8025–8046.

[22] Ralff MD, Kline CLB, Cukakisca OC, Wagner J, Lim B, Dicker DT, Prabhu VV, Oster W, and El-Deiry WS (2017). ONC201 demonstrates antitumor effects in both triple-negative and non–triple-negative breast cancers through TRAIL-Dependent and TRAIL-independent mechanisms. Mol Cancer Ther 16, 1290–1298.

[23] Kumar R, Riddle L, Griffin SA, Grundt P, Newman AH, and Rudd hedge RR (2009). Evaluation of the D3 dopamine receptor selective antagonist PG01037 on L-dopa-dependent abnormal involuntary movements in rats. Neuropharmacology 56, 944–955.

[24] Allen JE, Kline CL, Prabhu VV, Kim JH, and El-Deiry WS (2015). First-in-class small molecule ONC201 induces DR5 and cell death in tumor but not normal cells to provide a wide therapeutic index as an anti-cancer agent. PLoS One 10, e1413082.

[25] Madhukar N, Prabhu VV, Dardenne EE, Doherty F, VanEngelenburg A, Tarapore R, Garnett MJ, McDemott U, Benes C, and Oster W, et al (2017). Abstract 2792: The small molecule imipridone ONC201 is active in tumor types with dysregulated DRD2 pathway. Cancer Res 77.

[26] Tice MA, Hashemi T, Taylor LA, Duffy RA, and McQuade RD (1994). Characterization of the binding of SCH 39166 to the five cloned dopamine receptor subtypes. Pharmacol Biochem Behav 49, 567–571.

[27] Kang S, Dong SM, Kim BR, Park MS, Trink B, Byun HJ, and Rho SB (2012). Thioridazine induces apoptosis by targeting the PI3K/Akt/mTOR pathway in cervical and endometrial cancer cells. Apoptosis 17, 989–997.

[28] Gil-AI, Shafi A, Levkovitz Y, Nordenberg J, Taler M, Korov I, and Weizman A (2006). Phenothiazines induce apoptosis in a B16 mouse melanoma cell line and attenuate in vivo melanoma tumor growth. Oncol Rep 15, 107–112.

[29] Edagawa M, Kawauchi J, Hirata M, Goshima H, Inoue M, Okamoto T, Murakami A, Maehara Y, and Kitajima S (2014). Role of activating transcription factor 3 (ATF3) in endoplasmic reticulum (ER) stress–induced sensitization of p53-deficient human colon cancer cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)–mediated apoptosis through up-regulation of death receptor 5 (DR5) by zebularine and celecoxib. J Biol Chem 289, 21544–21561.

[30] Taketani K, Kawauchi J, Tanaka-Okamoto M, Ishizaki H, Tanaka Y, Sakai T, Miyoshi J, Machara Y, and Kitajima S (2012). Key role of ATF3 in p53-dependent DR5 induction upon DNA damage of human colon cancer cells. Oncogene 31, 2210–2221.

[31] Blasiak E, Lukasiewicz S, Szafarn-Plich K, and Dziezicka-Wasylewska M (2017). Genetic variants of dopamine D2 receptor impact heterodimerization with dopamine D1 receptor. Pharmacol Rep 69, 235–241.

[32] Singleton PA, Moss J, Karp DD, Atkins JT, and Janku F (2015). The mu opioid receptor: a new target for cancer therapy? Cancer 121, 2681–2688.

[33] Kulagowski JJ, Broughton HB, Curtis NR, Mawer JM, Ridgill MP, Baker R, Emmns F, Friedman SB, Woodrow R, and Patel S, et al (1996). 3-((4-(4-Chlorophenyl)piperazin-1-yl)-methyl)-1H-pyrrolo-2,3-b-pyridine: an antagonist with high affinity and selectivity for the human dopamine D4 receptor. J Med Chem 39, 1941–1942.

[34] Grundt P, Carson EE, Cao J, Bennett CJ, McElveen E, Taylor M, Floresca CZ, Choi JK, Jenkins BG, and Luedtke RR, et al (2005). Novel heterocyclic trans olefin portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2, 571–581.

[35] Leysen JE, Janssen PM, Megens AA, and Schotte A (1994). Risperidone: a novel antipsychotic with balanced serotonin-dopamine antagonism, receptor occupan-

[36] Seeman P, Corbet R, and Van Tol HH (1997). Asypical neuroleptics have low affinity for dopamine D2 receptors or are selective for D4 receptors. Neurropsychopharmacology 16, 93–110 [discussion 1-35].