Original Article
Small cell lung cancer and prostate cancer cells with varying neuroendocrine differentiation markers show sensitivity to imipridone ONC201/TIC10

Elizabeth Ding[1,2], Maximillian Pinho-Schwermann[1,2,3], Shengliang Zhang[1,2,3], Connor Purcell[1,2], Wafik S El-Deiry[1,2,3,4,5]

[1]Laboratory of Translational Oncology and Translational Cancer Therapeutics, The Warren Alpert Medical School of Brown University, Providence, RI 02903, USA; [2]Legorreta Cancer Center at Brown University, The Warren Alpert Medical School, Brown University, Providence, RI 02903, USA; [3]Department of Pathology and Laboratory Medicine, The Warren Alpert Medical School, Brown University, Providence, RI 02903, USA; [4]Division of Hematology-Oncology, Department of Medicine, Rhode Island Hospital and Brown University, Providence, RI 02903, USA; [5]The Joint Program in Cancer Biology, Brown University and The Lifespan Health System, Providence, RI 02903, USA

Abstract: Objectives: To investigate whether neuroendocrine differentiation (NED) markers, activation of the integrated stress response (ISR), and TRAIL pathway alter neuroendocrine tumor (NET) cell death and ONC201 sensitivity. Methods: We conducted cell viability assays to determine ONC201 sensitivity. Western blot analysis was performed to evaluate NED, ISR, and TRAIL pathway markers. Expression levels of NED markers were compared between cell lines with and without BRN2 overexpression. Results: Prostate cancer (PCa) and small cell lung cancer (SCLC) cell lines (N = 6) were sensitive to ONC201. Endogenous NET marker levels varied across PCa and SCLC cells. Transient BRN2 overexpression slightly reduced some NET markers while maintaining the sensitivity of PCa cells to ONC201. Conclusions: PCa cell lines exhibit sensitivity to ONC201, with variability of NED features. These findings are relevant to the design of future studies evaluating imipridone efficacy in PCa and suggest that non-NET patients could be included in such studies.

Keywords: Prostate cancer, small cell lung cancer, neuroendocrine differentiation, ONC201, SOX2, BRN2

Introduction

Prostate cancer (PCa) is the second most common malignancy and the leading cause of cancer-related death in men [1]. Androgen deprivation therapy (ADT) remains a cornerstone treatment for metastatic PCa patients by suppressing androgen receptor (AR) signaling [2]. ADT is effective in approximately 90% of patients, as evidenced by decreased prostate-specific antigen (PSA) levels [3]. While localized prostate cancer can be removed or treated successfully, treatments for advanced metastatic prostate cancer remain challenging [4].

After an average of 2-3 years, prostate cancer progresses to castration-resistant prostate cancer (CRPC) [3]. Reactivation of AR signaling occurs despite low serum androgen, driving disease progression and metastasis [2]. For patients with advanced metastatic castration-resistant prostate cancer (mCRPC), the prognosis is poor, with a mean survival time of ~25.6 months from diagnosis [5]. Treatments for mCRPC have expanded to include second-generation AR pathway inhibitors (API), such as enzalutamide (ENZ) and abiraterone (ABI), which further suppress AR signaling [3]. While these therapies improve overall patient survival [6], resistance to APIs invariably develops, and curative treatments remain rare [2, 7].

API-resistant prostate adenocarcinomas represent a clinical challenge due to the lack of effective third-line therapies and their potential to transform into lethal neuroendocrine prostate cancer (NEPC) through neuroendocrine differentiation (NED) [2]. The neural transcription fac-

tor BRN2 is a master regulator of NED and NEPC development *in vitro*, promoting NE marker expression and CRPC progression [8]. Previous studies have shown that AR directly represses BRN2, which affects BRN2 regulation of SOX2 [2], an additional NEPC-related transcription factor [9].

Only 0.5-2% of NEPC arise *de novo*, while approximately 25% of advanced PCa develop into NEPC through trans-differentiation of prostate adenocarcinoma, CRPC cells, or cancer stem cells as a result of therapy resistance [10]. Through lineage plasticity, PCa cells acquire neuroendocrine cell characteristics, such as the expression of neuroendocrine markers including chromogranin A (CgA) [11], PGP9.5/UCHL1 [12], synaptophysin (syp), BRN2, enolase 2 (Enol-2), and others [10]. These patients often present with low prostate-specific antigen (PSA) levels due to AR signaling loss [13]. NEPC patients face poor prognoses due to high metastatic burden, limited therapeutic options, and a less than 20% five-year survival rate [6]. Thus, there is a great need for effective therapies for high-risk NEPC.

Dordaviprone (ONC201) is a first-in-class imipridone initially discovered as a tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-inducing compound, TIC10. It was later found to activate the integrated stress response (ISR) [14] via ATF4/CHOP [15], inhibit Akt/ERK signaling, and induce cell death through pro-apoptotic death receptor 5 (DR5) receptor activation [14]. ONC201 acts as an agonist of mitochondrial caseinolytic protease (ClpP) [16], impairing oxidative phosphorylation and causing mitochondrial disruption and cell death [14]. Additionally, ONC201 functions as an antagonist of G-protein-coupled receptors (GPCR) and dopamine receptors D2 (DRD2) and D3 (DRD3) [14]. ONC201 demonstrates activityagainstvariousmalignanciesincludingH3K-27M mutated glioma and neuroendocrine tumors (NETs) with high DRD2 expression [17]. A recent phase II clinical trial study of ONC201 in neuroendocrine tumors with high DRD2 expression, including pheochromocytoma-paraganglioma (PC-PG) and desmoplastic small round cell tumor (DRSCT), demonstrated clinical efficacy and was well-tolerated by patients [18].

This study examines the hypothesis that markers of NED, activation of the integrated stress response (ISR) and the TRAIL pathway, as well as ClpP expression, contribute to neuroendocrine tumor cell death and sensitivity to ONC201/TIC10. We determined the sensitivity of small cell lung cancer (SCLC), a neuroendocrine tumor, and PCa to ONC201. Additionally, we proposed a model characterizing PCa and SCLC cell lines along a neuroendocrine tumor spectrum based on basal NED marker expression. Finally, we investigated whether BRN2 overexpression altered NED marker expression or sensitivity to ONC201. Our findings provide the background for advancing ONC201 into clinical studies as a potential therapy for NEPC and NETs.

Materials and methods

Reagents

ONC201 was provided by Oncoceutics/Chimerix, Inc. (Philadelphia, PA, USA) and stored at -20°C at a concentration of 20 mM.

Cell culture

Prostate cancer cell lines included PC3, DU145, LNCaP, and 22RV1. Small cell lung cancer cells included H1417 and H1048. All cell lines were acquired from the American Type Culture Collection (ATCC). PC3, DU145, 22RV1, LNCaP, and H1417 were cultured in RPMI-1640 medium (Cytiva SH30027.FS) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). H1048 cells were cultured in DMEM medium supplemented with 10% FBS and 1% P/S. All cell lines were incubated at 37°C with 5% CO_2. Cells were trypsinized with 0.25% trypsin when confluent. Cell line authentication and mycoplasma testing was done using PCR testing.

Cell viability assays

Cells were plated at a density of 3,000 cells per well in a 96-well plate with 100 µL of medium. After 24 hours, 20 µL of ONC201 was added in a 1:2 serial dilution from 20 to 0 µM and then incubated for 72 hours. 20 µL of CellTiterGlo bioluminescence agent was added to each well, and plates were mixed on a plate shaker for 3 minutes on a plate shaker to begin cell lysis. Cell viability was measured using a

CellTiterGlo assay, and bioluminescence images were collected with the Xenogen IVIS imaging system. Dose-response curves and IC_{50} doses were determined and graphed using GraphPad Prism.

Colony formation assays

After optimizing experimental conditions for the different cell lines, DU145 and 22RV1 were plated at a density of 200 cells per well in a 12-well plate. PC3 cells were plated at a density of 200 cells per well in a 6-well plate. After 24 hours, cells were treated with a range of doses of ONC201. DU145 and 22RV1 were treated with 1.33, 1.67, and 2 µM ONC201. PC3 cells were treated with 2, 4, and 6 µM ONC201. After 7 days of incubation, wells were washed with phosphate-buffered saline (PBS), fixed with formalin for 30 minutes, and stained with Coomassie Brilliant blue for 20 minutes. Colonies were counted, and the percent area was determined using ImageJ.

Western blots

Neuroendocrine differentiation markers, BRN2/SOX2 transcription factors, and TRAIL pathway proteins in prostate cancer and small cell lung cancer cell lines were examined using a western blot. Around 500,000 cells were plated and incubated in a 6-well plate for 24 hours for cell adherence. Cells were treated with ONC201 at IC_{50} drug dose and incubated at different time points (12, 24, and 48 hours). After cells were harvested, they were washed with cold PBS and lysed with RIPA buffer containing a protease inhibitor (Cell Signaling Technology 9806S) and phosphatase inhibitor (PHOSS-RO Roche 4906845001). Protein quantification was performed with Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA). Samples were boiled at 95°C for 10 minutes after a denaturing sample buffer was added. Cell lysates were electrophoresed through 4-12% SDS-PAGE gels (Invitrogen) and transferred to a PVDF membrane. Membranes were blocked with 5% milk in 1× TTBS for 1 hour and incubated overnight at 4°C with primary antibodies in 5% non-fat milk. Primary antibodies utilized include CgA (Abcam ab283265), CLpP (Cell Signaling 14181S), CLpX (Abcam ab168338), synaptophysin (Cell Signaling 5461), vinculin (Cell Signaling 4650), SOX2 (Cell Signaling 2748S), BRN2 (Cell Signaling 12137S), UCHL1/

PGP9.5 (Cell Signaling 13179), Enolase 2 (Cell Signaling 9536S), Androgen Receptor (Cell Signaling 5153S), DR5 (Cell Signaling 8074S), Ran (Cell Signaling 4462), Beta-Actin (Cell Signaling 4970), cPARP (Cell Signaling 5625), and ATF4 (Cell Signaling 11815). Primary antibodies were washed and incubated in appropriate HRP-conjugated secondary antibodies at a 1:10,000 dilution in 5% non-fat milk (rabbit, ThermoFisher Scientific, or mouse, ThermoFisher Scientific) for two hours. ECL western blotting detection reagent and Syngene imaging were used to measure protein expression levels.

Transient overexpression

POU3F2-Human_pcDNA3.1(+)-P2A-eGFP plasmid was created from GenScript. Bacteria containing the plasmid from agar stabs were streaked onto LB agar Petri dishes with 100 µg/mL ampicillin and grown at 30°C for 24 hours. Single colonies were midi-prepped (QIAGEN Plasmid Plus Midi Kit 12943) based on the manufacturer's protocol. Whole plasmid sequencing was done to confirm the plasmid sequence (Plasmidsaurus).

Statistical analysis

GraphPad Prism Version 10.2.2 was used for statistical analysis. Cell viability and colony formation assays were reported as mean ± SD. ONC201 dose-response curves were created with GraphPad. Cell viability assays and western blot analyses were conducted in replicates of three. Colony formation assays were conducted in replicates of nine. Treatment groups for colony formation assays were analyzed with a one-way ANOVA and t-tests with $P < 0.05$ considered significant.

Results

ONC201 is cytotoxic for PCa and SCLC cell lines

To understand the sensitivity of TRAIL-inducing ONC201 therapeutics in cell lines, we conducted a cell viability assay in six (N = 6) prostate and small cell lung cancer cell lines. A loss of viability was observed across various drug concentrations, with all cell lines showing decreased viability at low therapeutic doses (**Figure 1A**). Increasing ONC201 concentrations resulted in further reductions in cell viabil-
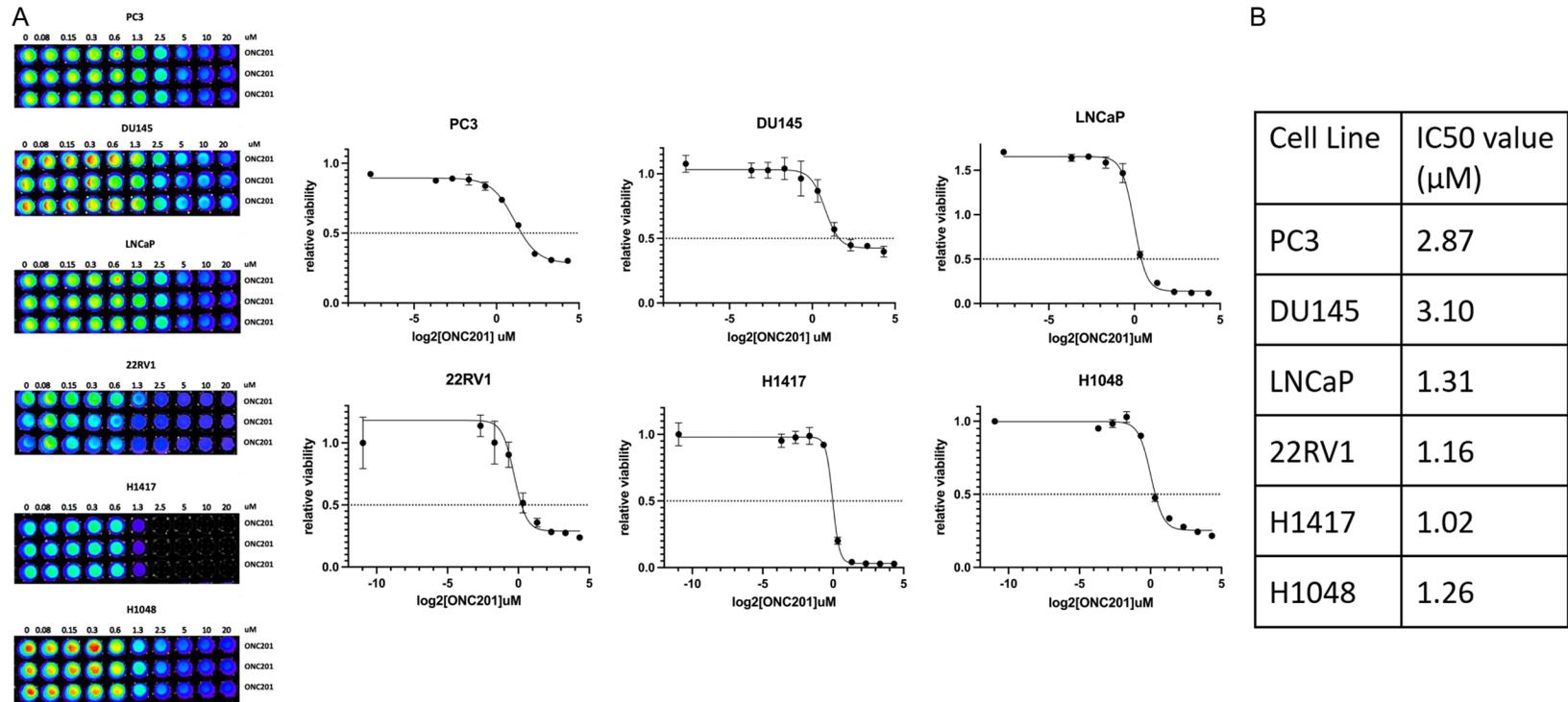
Figure 1. IC$_{50}$ curves for PCa and SCLC cell lines with ONC201 treatment. A. PCa and SCLC cell viability after 72 h of ONC201 treatment measured with CellTiterGlo (CTG) reagent. IC$_{50}$ curves of cell lines (right). Representative images of cell viability (left). B. Reported IC$_{50}$ values of cell lines treated with ONC201. PCa: Prostate cancer; SCLC: small cell lung cancer.

A

1 = PC3
2 = DU145
3 = LNCaP
4 = 22RV1
5 = H1417
6 = H1048
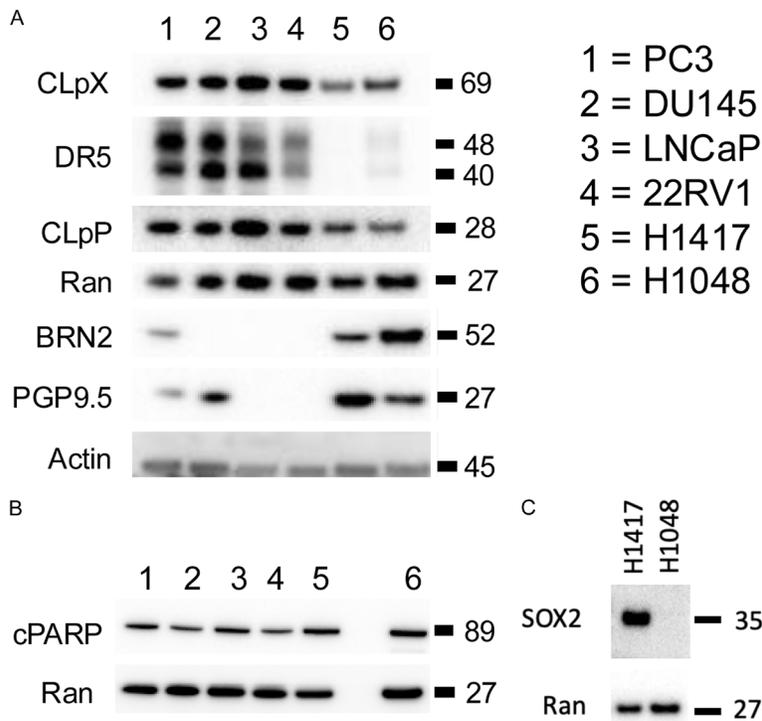
Figure 2. Basal expression levels of NED and TRAIL markers in PCa and SCLC. A. Western blot of endogenous TRAIL and NED marker expression in PCa and SCLC. B. Western blot of cPARP expression in PCa and SCLC. C. Western blot of SOX2 marker expression in SCLC. For all cell lines, membranes were collected from the same set of lysates and probed with a loading control. Ran and actin were used as loading controls. NED: neuroendocrine differentiation; PCa: Prostate cancer; SCLC: small cell lung cancer.

In PC3, low levels of BRN2 and PGP9.5 were observed. In DU145, BRN2 was not expressed, but high levels of PGP9.5 were detected. In LNCaP and 22RV1, neither BRN2 nor PGP9.5 was expressed (**Figure 2A**). Among SCLC, H1417 expressed high levels of SOX2, BRN2, and the highest observed levels of PGP9.5. In H1048, SOX2 was not present, but BRN2 and PGP9.5 were present, with the highest BRN2 levels among all other tested cell lines (**Figure 2A**, **2C**). These results provide a comprehensive analysis of basal levels of NE marker and TRAIL pathway protein expression in PCa and SCLC.

Based on protein expression levels of NED markers existing in the literature, we proposed a NED spectrum model for our cell lines (**Figure 3**). 22RV1 and LNCaP, which are androgen receptor-constitutive and androgen receptor-dependent, respectively, were classified as non-neuroendocrine cell lines. DU145 and PC3 showed partial NED markers expression and were categorized as being on the NE spectrum. H1048, H1417, and NCI-H660 were classified as neuroendocrine cell lines. NCI-H660 NED marker information was based on the literature [2, 19].

ONC201 treatment upregulates ATF4, cPARP, and DR5 and downregulates ClpX expression

To investigate the mechanistic pathways underlying ONC201-induced cell death, we conducted time-point western blot analyses (**Figure 4**). In DU145, an increase in DR5 expression increased at around 48 hours, while cleaved PARP (cPARP) expression increased at 24 hours, indicating cell death. The chaperone subunit ClpX, which regulates mitochondrial Clp protease, showed reduced expression at 12 and 24 hours, suggesting ONC201 induces cell apoptosis through mitochondrial proteolysis. ATF4 expression began increasing at 12 hours, with a significant increase observed at 24

ity in a dose-dependent manner. The most sensitive cell lines among the panel were H1417, a small cell lung cancer cell line (IC$_{50}$ = 1.02 µM), and 22RV1, a prostate cancer cell line (IC$_{50}$ = 1.16 µM), followed by H1048 (IC$_{50}$ = 1.26 µM) and LNCaP (IC$_{50}$ = 1.31 µM) (**Figure 1B**). These results demonstrate that PCa and SCLC cell lines exhibit sensitivity to ONC201 at low doses.

Characterization of NED spectrum model from endogenous NED marker expression in cell lines

To investigate the presence and endogenous neuroendocrine (NE) markers in PCa and SCLC, we performed western blots on solid tumor cell lines, examining the NED markers SOX2, PGP9.5, and BRN2 (**Figure 2A**, **2C**). Additionally, we analyzed endogenous levels of TRAIL pathway protein expression levels, including ClpX, ClpP, DR5, and cPARP (**Figure 2A**, **2B**). Endogenous levels of NE features were summarized (**Table 1**).

Table 1. Summary of cell lines and their neuroendocrine features

NED Marker	PC3	DU145	LNCaP	22RV1	H1417	H1048
BRN2	Low levels	ND	ND	ND	Low levels	High levels
SOX2	Low levels	Low levels	Low Levels	Low levels	High levels	?
Chromogranin A (CgA)	ND	ND	ND	ND	High levels	?
PGP9.5/UCHL1	Low levels	High levels	ND	ND	High levels	Low levels
Enolase 2/NSE	ND	Low levels	High levels	High levels	?	?
Androgen Receptor	ND	ND	High levels	ND*	?	?

Protein expression levels were detected through western blotting. NED: neuroendocrine differentiation; ND: not detected; ?: not tested. *22RV1 expresses AR receptor, but due to the splice site mutation, was not visible in the western blot.
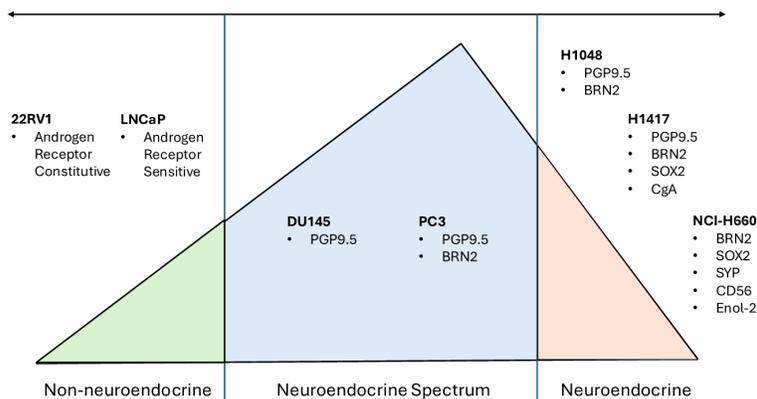
Figure 3. Characterization of NED spectrum model in PCa, SCLC, and NET. Characterization was based on basal neuroendocrine differentiation marker expression levels and scientific literature. NED: neuroendocrine differentiation; PCa: Prostate cancer; SCLC: small cell lung cancer.

In DU145, BRN2 overexpression increased the sensitivity to ONC201, with a lower IC_{50} value compared to the control vector (control vector IC_{50} = 4.46 µM; BRN2 overexpressed vector IC_{50} = 3.85 µM). PC3 and LNCaP showed decreased sensitivity to ONC201 following BRN2 overexpression. These results indicate heterogeneity among cell lines, with no meaningful change in ONC201 sensitivity.

Short-term BRN2 overexpression decreases SOX2 protein expression

hours, indicating the activation of the ISR and tumor cell death.

In 22RV1, DR5 expression increased at 24 hours and 48 hours. cPARP expression increased at 24 hours, and ClpX showed a slight decrease in expression at 12 hours. ATF4 expression increased at 12 hours, reaching high levels at 48 hours. These findings indicate ONC201 induces cell death through TRAIL pathway activation at varying time points.

Short-term overexpression of BRN2 alters PCa sensitivity to ONC201

To examine changes in sensitivity to ONC201 following BRN2 overexpression, we transiently overexpressed it in PC3, DU145, 22RV1, and LNCaP cell lines. Cells were treated with various doses of ONC201 and imaged after 72 hours (**Figure 5; Table 2**). BRN2 overexpression in 22RV1 resulted in a slight increase in IC_{50} values (control vector IC_{50} = 1.34 µM; BRN2 overexpressed vector IC_{50} = 1.91 µM).

We conducted western blot analyses in prostate cancer cell lines with transient BRN2 overexpression at 48 hours (**Figure 6**) to determine whether BRN2 overexpression would alter neuroendocrine marker levels. In PC3, BRN2 overexpression did not alter Enol-2, PGP9.5, or SOX2. Interestingly, in DU145 cell lines, BRN2 overexpression led to decreased expression of Enol-2 and PGP9.5. In LNCaP and 22RV1, we observed no change in Enol-2, and PGP9.5 expression. However, SOX2 expression decreased in LNCaP, while no changes were observed in 22RV1. Overall, NE marker protein expression changes following transient BRN2 overexpression at 48 hours vary among PCa lines.

Transient BRN2 overexpression with ONC201 treatment alters ClpX, DR5, and BRN2 expression

For DU145, we observed a decrease in ClpX expression when ONC201 was added at approximately 48 hours (**Figure 7**). Additionally, we observed an increase in DR5 expression in
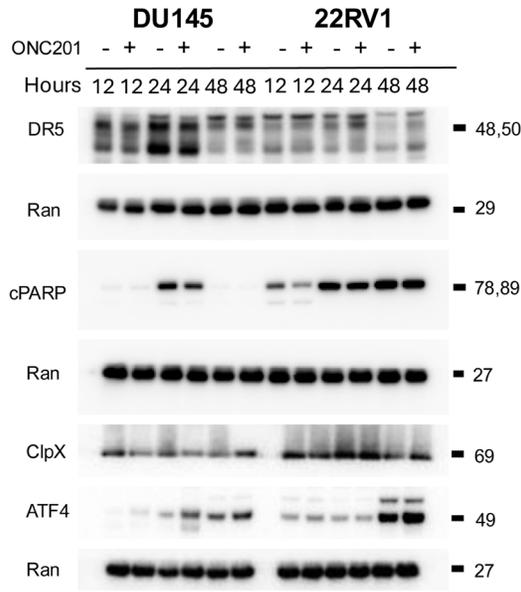
Figure 4. Early expression of TRAIL pathway proteins in response to ONC201. DU145 was treated with the IC$_{50}$ value of 3.10 µM ONC201, and 22RV1 was treated with the IC$_{50}$ value of 1.16 µM ONC201. DU145 and 22RV1 were treated with ONC201 for 12, 24, and 48 h. All membranes were collected from the same set of lysates and probed with a loading control.

both DU145 and LNCaP following ONC201 treatment. No significant changes were detected in PGP9.5 protein expression levels in DU145 when BRN2 was overexpressed. Interestingly, in both DU145 and LNCaP cells with transient BRN2 overexpression, we observed an increase in BRN2 protein expression levels when treated with ONC201. These findings indicate that transient BRN2 overexpression at 48 hours, combined with ONC201 treatment, alters the expression of NED marker BRN2 and TRAIL pathway proteins ClpX and DR5 in DU145 and LNCaP cells.

PCa colony forming ability decreases with ONC201 treatment

In addition to cell viability assays, we investigated whether similar sensitivity and response occurred in prostate cancer cell lines using long-term colony formation assays (**Figure 8**). Our data demonstrate that PC3, DU145, and 22RV1 are sensitive to ONC201, further supporting the conclusion that these cell lines are sensitive to ONC201 at low doses. PC3 cells showed a significant decrease in colony-form-

ing ability at 2.00 µM, with one-way ANOVA tests showing *p*-values of less than 0.0001 (**Table 3**). Similarly, DU145 and 22RV1 cells exhibited a significant reduction in colony-forming ability at 1.67 µM, with one-way ANOVA tests showing *p*-values also less than 0.0001 (**Table 3**). These results demonstrate that ONC201 effectively suppresses prostate cancer cell line growth, as observed by colony forming assays.

Discussion

Due to CRPC being resistant to APIs, the potential for development of highly aggressive NEPC tumors, and the lack of effective therapies, there is an urgent need to investigate novel therapeutics [19]. Neuroendocrine differentiation remains a complex process that is not yet fully understood, owing to its many underlying mechanisms, tumor heterogeneity, and various regulatory transcription factors [20]. Previous clinical trials using imipridones, specifically ONC201, demonstrated efficacy in PC-PG and DRSCT tumors, as well as in several other tumor types [18]. This study investigated whether PCa, CRPC, and SCLC cell lines are sensitive to ONC201. Additionally, we overexpressed BRN2, a driver of NED [2], in PCa to induce NEPC-like characteristics. Our findings reveal new insights with implications for CRPC and NEPC patients.

Our studies show that certain PCa cell lines, such as PC3, DU145, and H1417, exhibit greater neuroendocrine differentiation characteristics than others (**Table 2**). However, all tested cell lines (N = 6) demonstrated sensitivity to ONC201 at low doses, suggesting that ONC201 is effective against both PCa and SCLC regardless of their NED status (**Figure 1**). Supporting this, colony-forming ability of prostate cancer cell lines PC3, DU145, and 22RV1 was significantly reduced following ONC201 treatment (**Figure 8**; **Table 3**).

We propose a model for the spectrum of NED, which captures the complexity of NED in PCa and SCLC. Commonly, individual markers such as CgA, NSE, and syp are used to identify NE cells in tissue specimens via immunohistochemistry [21]. However, limitations in the accuracy of this method warrants the use of multiple biomarkers to improve diagnostic precision [21]. Our model incorporates several
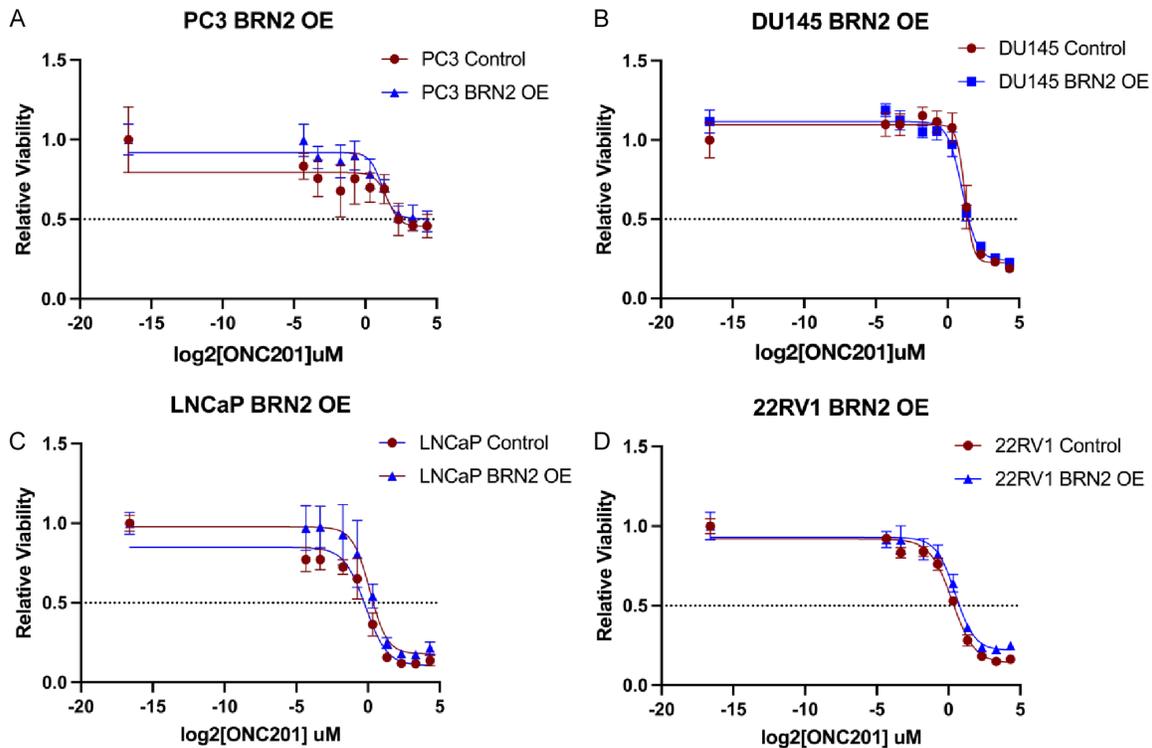
Figure 5. Transient overexpression of BRN2 alters PCa sensitivity to ONC201. A-D. Dose-response curves for 72 h ONC201 treatment after BRN2 plasmid overexpression compared to the control plasmid in PC3 (top left), DU145 (top right), LNCaP (bottom left), and 22RV1 (bottom right) cell lines. BRN2 was transiently overexpressed in cells using a 0.8 ug/uL BRN2 plasmid and 0.8 ug/uL pcDNA3.1 eGFP control plasmid for 48 h before ONC201 treatment for 72 h. Cell viability was measured through the addition of CellTiterGlo (CTG) reagent. PCa: Prostate cancer.

Table 2. IC_{50} values of PCa lines treated with varying doses of ONC201 with and without transient BRN2 overexpression

Cell Line	BRN2 Control IC_{50} (μM)	BRN2 OE IC_{50} (μM)
PC3	4.66	8.51
DU145	4.46	3.85
LNCaP	0.78	1.47
22RV1	1.34	1.91
H1417	N/A	N/A
H1048	N/A	N/A

PCa: Prostate cancer.

markers to better delineate where cell lines lie on the NED spectrum (**Figure 3**). The characteristics that define the neuroendocrine phenotype remain incompletely understood, necessitating further research to characterize differentiation-associated phenotypes.

Regarding changes in sensitivity to ONC201 after BRN2 overexpression for 48 hours, we observed no significant changes in sensitivity

levels across cell lines. However, there was some heterogeneity, with DU145 exhibiting a slight increase in sensitivity, while LNCaP, PC3, and 22RV1 showed slight decreases (**Table 2**). The underlying reasons for these variations remain unclear and require further investigation.

Interestingly, BRN2 overexpression did not lead to significant increases in the expression of tested NED markers. In 22RV1 and LNCaP, markers such Enol-2 and PGP9.5 showed no changes (**Figure 6**). Notably, we observed a decrease in SOX2 expression in LNCaP. While we hypothesized that BRN2 overexpression would increase SOX2 expression, SOX2 regulation in PCa is still an elusive matter [2]. Further research is necessary to confirm the relationship between BRN2 and SOX2 and to elucidate the downstream pathways because of their interplay. Downstream mechanistic pathway impacts from SOX2 and BRN2 interplay continue to remain unclear and to be explored [2]. Other transcription factors such as ASCL1, NEUROD1, BRN4, and SOX2, have been identi-
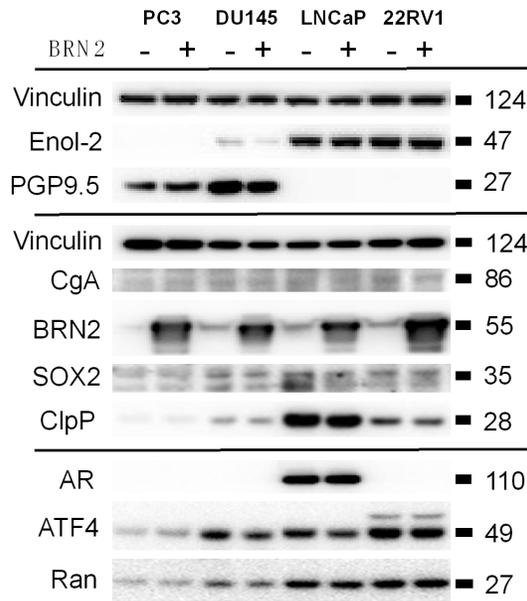
Figure 6. Overexpression of BRN2 alters NED marker expression. PCa cell lines were transiently overexpressed with 0.8 µg/µL BRN2 plasmid and 0.8 µg/µL pcDNA3.1 eGFP control plasmid for 48 h. Membranes were probed with a loading control. All membranes were analyzed from one set of lysates. NED: neuroendocrine differentiation; PCa: Prostate cancer.

Figure 7. BRN2 overexpression in combination with ONC201 treatment alters NED marker and TRAIL pathway expression in DU145 and LNCaP. Cells were transfected with 0.8 µg/µL BRN2 plasmid and 0.8 µg/µL pcDNA3.1 eGFP control plasmid for 48 h before treating with IC_{50} doses of ONC201 for 48 h. The membrane was probed with a loading control and analyzed from one set of lysates. NED: neuroendocrine differentiation.

fied as lineage-defining factors in the NED process [22-25]. While this study focused on BRN2, further investigations into other transcription factors, as well as factors like tumor microenvironments (TME) and epigenetic modifiers, are critical for understanding the NED process [25, 26].

Our study has several limitations. First, there was a lack of *in vivo* experiments. Additionally, the transient overexpression of BRN2 using plasmids may not fully represent the NED process due to the short experimental timeframe. Finally, a limitation is the small number of cell lines tested; expanding this to include more cell lines would strengthen future analyses. Future directions include evaluating the effects of overexpressing SOX2 and other transcription factors on NED features and ONC201 sensitivity. We also plan to explore changes in neuroendocrine marker expression following siRNA-mediated knockdown of BRN2 and SOX2 in PCa and SCLC (Figure S1). Additionally, investigating the role of emerging NED markers, such as DLL3 and FOXA1, will further enhance our understanding of NED in PCa and SCLC and refine the NED spectrum model [27, 28].

Our data indicate that PCa and SCLC exhibit sensitivity to ONC201, regardless of the degree of NED features based on protein expression. While neuroendocrine tumors are sensitive to ONC201, our findings highlight that PCa with and without pronounced neuroendocrine characteristics responds to ONC201. These insights have important implications for future clinical trial design, evaluating imipridone efficacy in prostate cancer, and determining the eligibility of non-neuroendocrine cancer patients. Although significant progress has been made in understanding the NED process, we are still far from fully understanding mechanisms and pathways. Future research is essential to identify targets for therapeutic treatment.

Disclosure of conflict of interest

W.S.E-D. is a co-founder of Oncoceutics, Inc., a subsidiary of Chimerix. Dr. El-Deiry has disclosed his relationship with Oncoceutics/
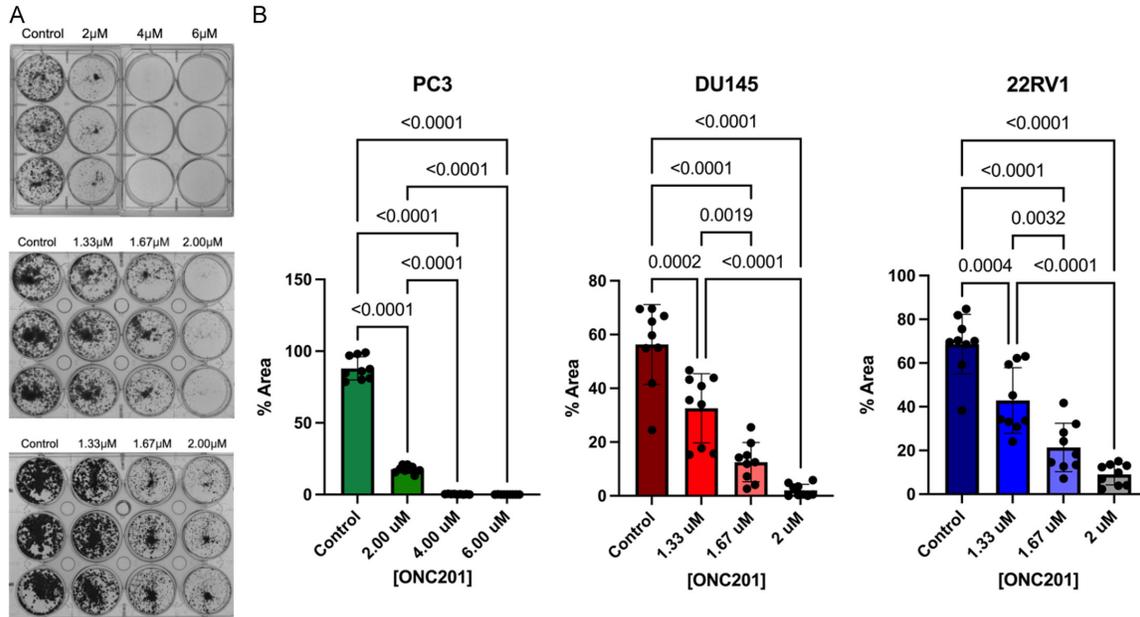
Figure 8. Colony formation assays of PCa cell lines following ONC201 treatment. A. Representative images of biological replicates after 7 days (top to bottom: PC3, DU145, 22RV1). B. Colony formation assay quantification for PC3, DU145, and 22RV1 cell lines (left to right) as mean ± SD. Treatment groups were compared using one-way ANOVA tests. PCa: Prostate cancer.

Table 3. Descriptive statistics showing the percent area of PCa lines when treated with varying doses of ONC201

Cell Line	Control (percent area)	Control to 1.33 μM	Control to 1.67 μM	Control to 2 μM	Control to 4 μM	Control to 6 μM
PC3	87.99% ± 8.02	N/A	N/A	< 0.0001	< 0.0001	< 0.0001
DU145	56.32% ± 14.87	0.0002	< 0.0001	< 0.0001	N/A	N/A
22RV1	68.64% ± 13.68	0.0004	< 0.0001	< 0.0001	N/A	N/A

PCa: Prostate cancer.

Chimerix and potential conflict of interest to his academic institution/employer. He is fully compliant with NIH and institutional policy managing this potential conflict of interest.

Address correspondence to: Wafik S El-Deiry, Laboratory of Translational Oncology and Translational Cancer Therapeutics, The Warren Alpert Medical School of Brown University, Providence, RI 02903, USA. E-mail: wafik@brown.edu

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Supplementary materials

Preliminary siRNA knockdown at 5, 10, and 20 pmol decreases BRN2 expression in PC3

To perform future BRN2 knockdown experiments, we conducted an optimization experiment on PC3 cells to investigate a concentration of siRNA that would result in significant BRN2 protein expression decrease. We observed a decrease in BRN2 protein expression in all concentrations of siBRN2 (Figure S1).
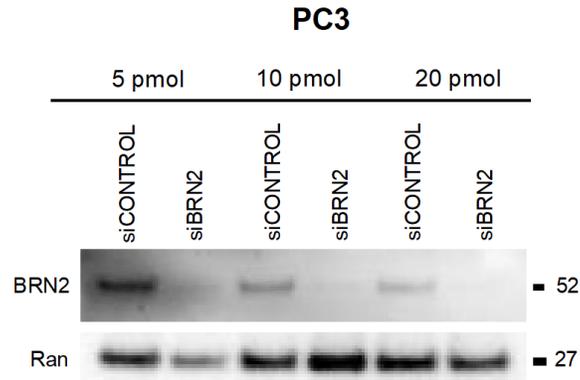
Figure S1. Optimization of BRN2 siRNA concentrations in PC3. PC3 were transfected with 5, 10, or 20 pmol of siBRN2 or siCONTROL for 48 h. The membrane was probed with a loading control and analyzed from one set of lysates.