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Original Article

Reduced EZH1/2 expression in imipridone-treated cells correlates with synergy following combinations with EZH1/2 or HDAC inhibitors in diffuse glioma and other tumors

Yiqun Zhang[1,2,3,4], Kelsey E Huntington[1,2,3,4], Attila A Seyhan[1,2,3,4], Nikos Tapinos[2,3,5], Rishi R Lulla[1,2,6], Michelle Monje[7], Eric T Wong[2,3,8,9], Clark C Chen[2,3,5], Wafik S El-Deiry[1,2,3,4,7,9]

[1]Laboratory of Translational Oncology and Translational Cancer Therapeutics, Warren Alpert Medical School of Brown University, Providence, RI, USA; [2]Legorreta Cancer Center, Brown University, Providence, RI, USA; [3]Joint Program in Cancer Biology, Brown University and Lifespan Cancer Institute, Providence, RI, USA; [4]Department of Pathology and Laboratory Medicine, Brown University, Providence, RI, USA; [5]Department of Neurosurgery, Lifespan and Brown University, Providence, RI, USA; [6]Division of Pediatric Hematology/Oncology, Department of Pediatrics, Brown University, Providence, RI, USA; [7]Department of Neurology and Neurological Sciences, Pediatrics, Pathology, Neurosurgery, The Howard Hughes Medical Institute, Stanford University, Palo Alto, CA, USA; [8]Department of Medicine, Radiation Oncology, Neurosurgery and Neurology, Brown University, Providence, RI, USA; [9]Division of Hematology/Oncology, Department of Medicine, Lifespan and Brown University, Providence, RI, USA

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Abstract: Small molecule imipridones including ONC201, ONC206 and ONC212 have anti-cancer activity mediated in part through the integrated stress response, induction of TRAIL and its receptor DR5, and activation of mitochondrial caseinolytic protease ClpP with impaired oxidative phosphorylation. ONC201 provides clinical benefit in a subset of patients with histone H3K27M-mutated diffuse glioma (DG). We hypothesized that EZH2 inhibitors (EZH2i) may sensitize tumors to imipridones by mimicking H3K27M mutation. EZH1 is a homolog and alternative for EZH2 in assembling PRC2 complex. We combined ONC201, ONC206 or ONC212 plus dual EZH1/2i in tumors and observed synergy. We observed synergies with imipridones combined with HDACi or triple combination of ONC201/ONC206, EZH2i and HDACi in DG, GBM, prostate cancer and SCLC cells. Our observations implicate EZH1/2 suppression in mechanism of anti-cancer effect of imipridones. We investigated effects of imipridones on EZH1/2 in DG cells and solid tumor cells including GBM, CRC, PDAC, SCLC, prostate cancer, gastric cancer, HCC and breast cancer cells and found inhibition of EZH1/EZH2 expression across tumor types and cell viability suppression by imipridones is correlated with EZH1/2 reduction. Imipridone or EZH2i-treated tumor cells showed similar cytokine profile changes. RNA-seq showed ONC201 and EHZ2i tazemetostat-treated cells have similar transcriptional profiles and share overlap of top regulated genes. Thus, imipridones inhibit EZH1/2 in tumor cells in a manner that mimics H3K27M mutation supporting their role in anti-cancer efficacy. ONC201 and EZH2i share similar targets and actions on tumors. Synergistic combinations of imipridones plus EZH1/2i or imipridones, EZH2i and HDACi merit further investigation.

Keywords: ONC201, ONC206, ONC212, EZH1, EZH2, glioma, DMG, H3K27M, tazemetostat, ISRIB, vorinostat, Panobinostat, valemetostat, cytokine profiles, epigenetic therapy, solid tumor

Introduction

ONC201 was discovered as an anti-cancer compound in the screen for p53-independent inducers of the TNF-related-apoptosis-inducing ligand (TRAIL) gene transcription [1-3]. It is the founding member of the imipridone family including another two members currently under development, ONC206 and ONC212. Imipridones exert a tumoricidal function through induction of TRAIL [2, 3], activation of an HRI-dependent eIF2-alpha- and ATF4/CHOP-mediated integrated stress response with downstream induction of TRAIL receptor, DR5

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[4]. These events are triggered by imipridone agonist action and activation of mitochondrial caseinolytic protease ClpP with consequent impairment of oxidative phosphorylation [5, 6].

DG (diffuse glioma) is typically an inoperable tumor with a poor prognosis requiring the development of novel therapies for cure. More than 80% of DG patients carry the driver H3K27M mutation leading to epigenomic reprogramming and oncogenesis [7]. ONC201 was observed to provide clinical benefit in a subset of DG patients with histone H3K27M mutation in some early clinical data [8, 9].

H3K27M mutation inhibits the function of EZH2 in methylating H3K27, thus reshaping the epigenetic profile [10]. EZH1 is a homolog of EZH2 and forms an alternative for EZH2 in assembling the PRC2 complex. Inhibition of EZH1/2 downregulates the H3K27me3 and derepressed target gene transcription, while histone acetylation leads to a more relaxed chromatin structure and ultimately gene transcription activation.

Based on the effect of imipridones on DG tumors carrying H3K27M mutation, we hypothesized that EZH2 inhibitor may sensitize such tumors to imipridones by mimicking H3K27M mutation. EZH2 inhibitors are effective in H3K27M-mutant DG, and the underlying mechanism may be a reduction of H3K27me3 in the tumors [11]. Dual inhibition of EZH1 and EZH2 was reported effective in some hematological malignancies in several preclinical studies [12-14].

These phenomena led us to investigate the action of imipridones on EZH1 and EZH2 protein expression and provide the rationale for the combination of imipridones plus EZH inhibitors or the triple combination of imipridones, EZH inhibitors and HDAC inhibitors in the treatment of tumors, especially given that we already observed synergy of ONC201 plus EZH2 inhibitor, ONC201 plus HDAC inhibitors or ONC201 plus EZH2 inhibitor and HDAC inhibitors in a panel of tumors in our previous studies [15].

We found in the present studies that imipridones reduce both EZH1 and EZH2 protein expression levels. This novel observation raised the question as to whether there are similarities between imipridones and EZH inhibitors with respect to their actions and molecular mechanisms in tumors. We performed RNA-seq and cytokine profile assays using tumor cells following treatment with imipridones or EZH2 inhibitor to look for the similarities.

Our results provide novel insights into the mechanism of action of imipridones and support the rationale for their combination with epigenetic drugs some of which are already clinically approved as glioma therapies.

Methods

Cell culture and reagents

All DG cell lines including SU-DIPG-4, SU-DIPG-13, SU-DIPG-25, SU-DIPG-29 and SU-DIPG-36 cell lines were provided by Dr. Michelle Monje at Stanford University and were generously shared by Dr. Tapinos with our group. The cells were maintained in Tumor Stem Medium (TSM) made of TSM Base enriched with B-27 supplement minus vitamin A, 0.2% Heparin (from STEMCELLTM Technologies, Vancouver, BC, Canada), human PDGF-BB, human PDGF-AA, human FGF-basic 154 aa (FGF2), and human EGF (from Shenandoah Biotechology Inc., Warwick, PA, USA). TSM base was made of 1:1 mixture of Neurobasal-A Medium and D-MEM/F-12 with Antibiotic-Antimycotic liquid, MEM Sodium Pyruvate Solution, non-essential amino acids solution, glutaMAX, HEPES Buffer solution (all purchased from Thermo Fisher Scientific Inc., Invitrogen brand, Carlsbad, CA, USA).

The human prostate cancer, PDAC, CRC, gastric cancer, SCLC, HCC, breast cancer and GBM cell lines were purchased from the American Type Culture Collection (ATCC). H1048 and H1882 SCLC cells were cultured in HITES medium [DMEM: F12 Medium supplemented with 0.005 mg/ml Insulin, 0.01 mg/ml Transferrin, 30 nM Sodium selenite (final conc.), 10 nM Hydrocortisone (final conc.), 10 nM beta-estradiol (final conc.), extra 2 mM L-glutamine (for final conc. of 4.5 mM) and 5% fetal bovine serum]. All the other cell lines were cultured in their ATCC-recommended media supplemented with 10% (v/v) fetal bovine serum and 1% Penicillin/Streptomycin. All cell lines were confirmed to be mycoplasma-free using PCR testing methods and cultured with or without chemotherapy agents at 37°C within a 95%

humidified atmosphere containing 5% carbon dioxide in an incubator.

Tazemetostat was purchased from Selleckchem and was solubilized in DMSO at a storage concentration of 20 mM. Valemetostat was purchased from Selleckchem and was solubilized in DMSO at a storage concentration of 10 mM. Vorinostat was purchased from MedKoo Biosciences and was solubilized in DMSO at a storage concentration of 50 mM. Panobinostat was purchased from MedKoo Biosciences Inc. and was solubilized in DMSA at a storage concentration of 20 mM.

ONC201, ONC206 and ONC212 were supplied by Chimerix, Inc. and reconstituted in DMSO at a storage concentration of 20 mM. Integrated stress response inhibitor (ISRIB) with the formula of C22H24Cl2N2O4 and the IUPAC name of trans-N, N'-(Cyclohexane-1,4-diyl)bis(2-(4-chlorophenoxy)acetamide), was purchased from Selleckchem and was solubilized in DMSO at a storage concentration of 20 mM.

Immunoblotting

Cells were seeded in 6-well plates at a density of 4-7×10^5 cells per well depending on the doubling time of the cells and incubated overnight in culture media before the addition of ONC201/ONC206/ONC212 alone or combination of ONC201/ONC206/ONC212 plus tazemetostat, ONC201/ONC206/ONC212 plus valemetostat, ONC201 plus vorinostat/panobinostat and tazemetostat or ONC206 plus panobinostat and tazemetostat. The culture was continued for 48 hours. Then, the cells were washed with PBS and lysed in lysis buffer [150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)]. The proteins were quantified with the Bio-Rad protein assay. LDS Sample Buffer (4X) and reducing reagent were added to the lysates. For the immunoblotting of histone, the cells were lysed directly with lysis buffer [150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)] plus LDS Sample Buffer (4X). The lysates were loaded equally onto 4 to 12% NuPAGE SDS-polyacrylamide gels (Thermo Fisher Scientific). Standard procedures were performed to transfer proteins to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% milk, the PVDF membranes were incubated with primary antibody overnight and subsequently appropriate secondary antibodies labeled with horseradish peroxidase for 1 hour. The membranes were developed using an ECL reagent. The primary antibodies used in this study were as follows: antibodies against EZH1 (D7D5D) (cat. no. 42088, Cell Signaling), EZH2 (D2C9) XP (cat. no. 5246, Cell Signaling), Cleaved PARP (Asp214) (19F4) (cat. no. 9546S, Cell Signaling), Histone H3 (tri-methyl K27) antibody (mAbcam 6002), β-Actin (A5441, Millipore Sigma), and Ran (cat. no. 610341, BD Bioscience). Secondary antibodies were acquired from Pierce (cat. nos. 31430 and 31460) (horseradish peroxide-conjugated).

Cell viability and apoptosis assays

Cells were seeded in opaque-walled 96-well plates at a density of 25,000 cells per well for H1882 and 5000 cells per well for all the other cells and incubated overnight in 100 µL culture medium before the addition of ONC201, ONC206 or ONC212 alone or combination of ONC201, ONC206 or ONC212 plus tazemetostat, ONC201, ONC206 or ONC212 plus valemetostat, ONC201 plus vorinostat or panobinostat and tazemetostat or ONC206 plus panobinostat and tazemetostat. After treatment for 72 hours. 20 µL CellTiterGlo bioluminescence agent (Promega Corporation, Madison, WI) was added to each well. The content was mixed for 2 minutes on a plate shaker to induce cell lysis. Cell viability was determined by the CellTiterGlo assay. Combination indices (CI) were calculated by the method of Chou and Talalay using the CompuSyn software. CI < 1.0 indicates drug synergy.

Reverse-transcription polymerase chain reaction (RT-PCR)

MDA-MB-468 breast cancer cells were seeded in 6-well plates at a density of 5×10^5 cells per well and incubated overnight in culture media before the addition of ONC201. Treatment was continued for 24 hours. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to manufacturers' instructions and quantitated using a Nanodrop spectrophotometer. Genes of interest were amplified using 2 µg of total RNA reverse-transcribed to cDNA using a Superscript II kit (Invitrogen) with random hexamer primer. In the RT-PCR step, PCR reactions were performed with 1 ml cDNA/20 ml reaction

and primers specific for EZH1 (F: 5'-caattcaagctggcgaagag-3', R: 5'-caagacagtgccgctacca-3'), EZH2 (F: 5'-gccaagagagccatccagac-3', R: 5'-ccgacatacttcagggcatca-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F: 5'-gcatcttcttttgcgtc-3', R: 5'-tgtaaaccatgtagttgaggt-3') with AccuPrime™ Pfx DNA Polymerase kit (ThermoFisher). Thermal cycling was initiated at 95°C for 10 min followed by 35 cycles of PCR (95°C for 15 s and 60°C for 1 min). GAPDH was used as an endogenous control. The PCR product was detected by electrophoresis on 3% agarose gels.

Cytokine profile analysis

Cells were plated at 2.5×10^4 cells in a 48-well plate in a complete medium and incubated at 37°C with 5% CO_2 overnight. The complete medium was replaced with a drug-containing medium until almost all the tumor cells were adherent to the bottom of the plates. Subsequently, the culture supernatants were collected after 48 hours of incubation and were frozen at -80°C until the measurement of cytokines was performed. On the day of analysis, samples were thawed and centrifuged to remove cellular debris. An R&D systems Human Premixed Multi-Analyte Kit (R&D Systems, Inc., Minneapolis, MN) was run on a Luminex 200 Instrument (LX200-XPON-RUO, Luminex Corporation, Austin, TX) according to the manufacturer's instructions. Cell culture supernatant levels of TNF-alpha, 4-1BB/TNFRSF9/CD137, IL-8/CXCL8, Ferritin, IFN-beta, IL-10, CCL2/JE/MCP-1, VEGF, CXCL13/BLC/BCA-1, IFN-gamma, CCL20/MIP-3 alpha, CCL3/MIP-1 alpha, CCL22/MDC, CCL4/MIP-1 beta, Fas Ligand/TNFSF6, IL-17/IL-17A, IL-2, BAFF/BLyS/TNFSF13B, GM-CSF, CXCL5/ENA-78, TRANCE/TNFSF11/RANK L, CXCL9/MIG, G-CSF, IFN-gamma R1/CD119, VEGFR3/Flt-4, C-Reactive Protein/CRP, CXCL11/I-TAC, IL-21, CXCL14/BRAK, IL-6, Fas/TNFRSF6/CD95, TRAIL R3/TNFRSF10C, IL-4, CCL5/RANTES, PD-L1/B7-H1, CCL7/MCP-3/MARC, Chitinase 3-like 1, CXCL10/IP-10/CRG-2, IL-1 beta/IL-1F2, IL-7, Prolactin, CCL8/MCP-2, TRAIL R2/TNFRSF10B, M-CSF, IL-15, Granzyme B, IFN-alpha, TREM-1, IL-12/IL-23 p40, TRAIL/TNFSF10, CCL11/Eotaxin, IL-18/IL-1F4 were measured. The means of triplicate values of treatment samples were compared with untreated samples by t-test. The cytokine profiles of different treated groups were clustered and depicted with R 4. 2. 2.

RNA-sequencing

SU-DIPG-13 and SU-DIPG-25 cells were seeded in 6-well plates at a density of 7×10^5 cells per well and incubated for 24 hours in culture media before treatment. All treatment conditions were collected in biological triplicate. The cells were treated with ONC201, Panobinostat or tazemetostat alone, or combination of ONC201 plus Panobinostat or ONC201 plus tazemetostat, or a triple combination of ONC201 plus Panobinostat and tazemetostat. Subsequently, the cells were collected after incubation for 12 hours and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to manufacturers' instructions and quantitated using a Nanodrop spectrophotometer. The RNA-sequencing was performed by AZENTA US Inc. Poly A selection was performed before sequencing. cDNA was synthesized. Sequencing depth was 20-30 million reads per sample. Standard RNA analysis package includes mapping, and differential gene expression. Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate *p*-values and log2 fold changes. Genes with an adjusted *p*-value < 0.05 and absolute log2 fold change > 1 were called differentially expressed genes. The transcriptional profile of ONC201, tazemetostat and Panobinostat treated DMG cells were clustered with R 4. 2. 2.

Results

Imipridones downregulate the expression of EZH1 and EZH2 in a panel of human cancer cell lines across tissue origins

To investigate the role of EZH1/2 in the mechanism of action of anti-cancer imipridone therapeutic agents, we performed immunoblotting for EZH1 and EZH2 after treating multiple human cancer cell lines including prostate cancer, PDAC, CRC, gastric cancer, SCLC, breast cancer, DMG and GBM cells with ONC201 or ONC206 or ONC212 for 48 hours. Both EZH1 and EZH2 were downregulated by ONC201 or ONC206 or ONC212 (**Figures 1** and S1). EZH1 and EZH2 are histone methyltransferases. They catalyze the addition of methyl groups on histone H3 at lysine. The decrease of EZH1 and EZH2 by imipridones brought up the question of whether the impridones can reduce methyla-

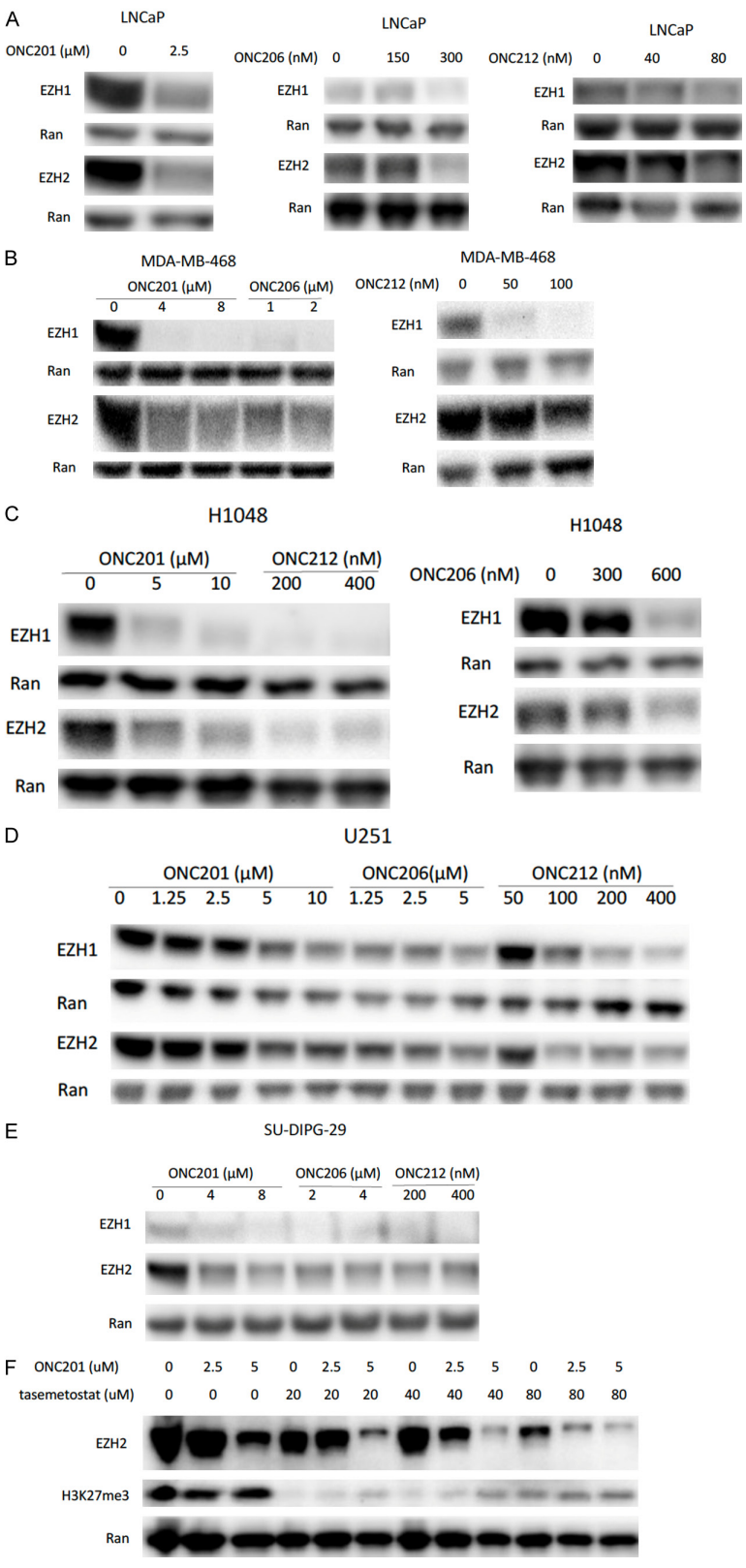


Figure 1. ONC201, ONC206 and ONC212 reduce EZH1 and EZH2 protein levels in tumor cells, and ONC201 can't reduce H3K27 trimethylation as tazemtostat does. Immunoblotting of EZH1 and EZH2 in (A) LNCaP prostate cancer, (B) MDA-MB-468 TNBC, (C) H1048 SCLC, (D) U251 GBM and (E) SU-DIPG-29 DMG cells upon the treatment with ONC201, ONC206 or ONC212. (F) Immunoblotting of EZH2 and H3K27me3 in MCF7 cells treated with ONC201 or tazemetostat for 72 hours showed that ONC201 reduce EZH2 but can't reduce H3K27me3, whereas tazemetostat reduce H3K27me3.

tion levels of H3K27. Thus, we tested the trimethylation level in MCF7 cells treated with ONC201. EZH1 and EZH2 proteins were reduced as expected and trimethylation of H3K27 can't be reduced by ONC201 (**Figure 1F**).

Given that imipridones induce the integrated stress response (ISR) in tumor cells with ATF4 upregulation as the marker and reduce global protein production, we suspected that the decrease of EZH1/2 may be attributable to the ISR. Thus, we treated U251 GBM cells with the combination of ISRIB and ONC201 or ONC206 to rescue the decrease of EZH1/2 by inhibiting ISR. However, the ISRIB could not rescue the decrease of EZH1/2 induced by ONC201 or ONC206 (**Figure 2**). Thus, the decrease of EZH1/2 expression induced by imipridones cannot be attributed to the ISR.

We assessed the suppression of cell viability by ONC201, ONC206, or ONC212 alone in the panel of tumor cells by CellTiterGlo assay after treating the cells with ONC201, ONC206, or ONC212 for 72 hours (Figure S2) and picked cell viability at doses with treatment of ONC201, ONC206 or ONC212 for each cell line (Table S1) to perform linear regression. The linear regression showed that the

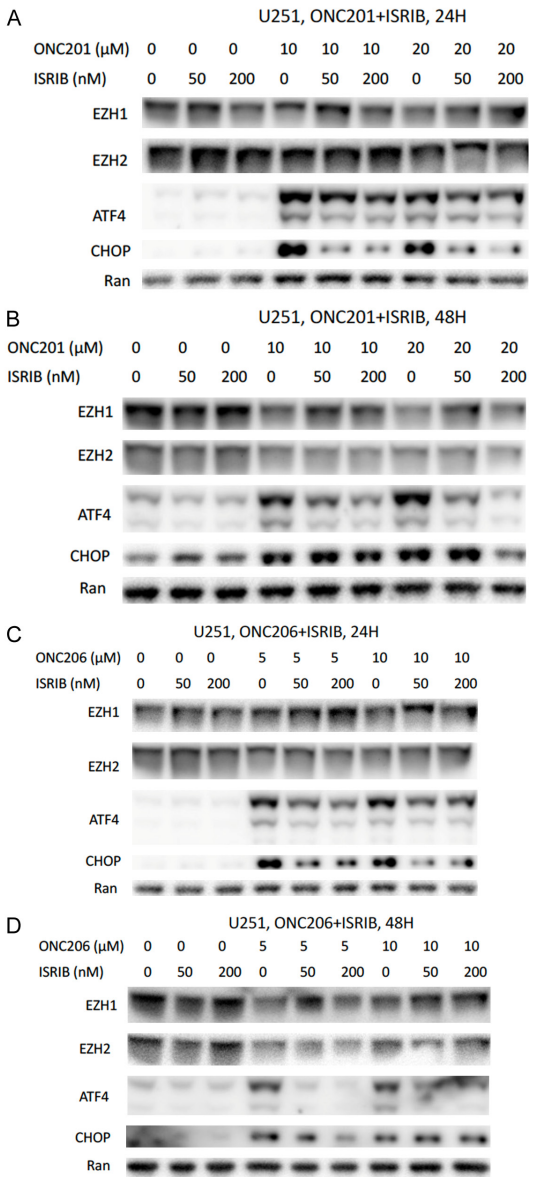


Figure 2. ISRIB does not rescue reduction of EZH1/2 following imipridone treatment. Immunoblotting of EZH1/2, ATF4 and CHOP in U251 cells upon treatment of ONC201, ISRIB or the combination of ONC201 and ISRIB for (A) 24 hours and (B) 48 hours, and ONC206, ISRIB or the combination of ONC206 and ISRIB for (C) 24 hours and (D) 48 hours.

cell viability suppression is correlated with EZH1 reduction and showed a trend to correlate with EZH2 reduction (**Figure 3**). In the assessment of EZH1 and EZH2 mRNA levels in MDA-MB-468 cells treated with ONC201 for 24 hours, ONC201 did not appear to reduce transcription of EZH1 or EZH2 (Figure S1O), while it decreased EZH1 and EZH2 protein expression in MDA-MB-468 (**Figure 1B**). RNA-sequencing of SU-DIPG-13 and SU-DIPG-25 cell lines treated with ONC201 showed no alteration of transcription of EZH1 or EZH2 (Figure S1P). Thus, EZH1 and EZH2 are downregulated by imipridones at the protein level.

H3K27 acetylation and reduction of EZH1 and EZH2 correlate with synergy when imipridones are combined with EZH1/2 inhibitors or HDAC inhibitors

EZH1/2 inhibition downregulates methylation of H3K27 and thereby leaves the H3K27 accessible to acetylation. EZH1 and EZH2 inhibition by imipridones provides a rationale for combining imipridones with EZH1/2 inhibitors and the addition of HDAC inhibitors in the combination of imipridones plus EZH1/2 inhibitors in the treatment of tumors.

We treated DMG cells with imipridones, tazemetostat, or HDAC inhibitors alone or combinations of imipridones plus tazemetostat, imipridones plus HDAC inhibitors, or the triple combination of imipridones plus tazemetostat and HDAC inhibitor, assessed the cell viability by CellTiterGlo assay after treatment, and quantified the synergy with combination index (CI). CI < 1 indicates synergy, and the lower the CI, the more potent the synergy. We observed synergies in DMG (**Figures 4A-D**, S3A-G; Table S2) GBM (Figure S3H-K; Table S2), prostate cancer (**Figures 4E-G**, S3L-N; Table S2), liver cancer (Figure S3O; Table S2) and SCLC (Figure S3P-S; Table S2) cells. The cell line experiments revealed synergies with the combinatorial treatments and the synergies are listed in **Table 1**. Immunoblotting of cleaved-PARP demonstrated more apoptosis with a combination of valemetostat plus ONC201, ONC206, or ONC201 (**Figure 4H-J**) in 22Rv1 prostate cancer cells, combination of ONC206 plus valemetostat in H1048 SCLC cells (**Figure 4K**), combination of ONC206 plus Panobinostat and tazemetostat in SU-DIPG-25 DMG cells (Figure S3T), combination of ONC201 plus valemetostat in LNCaP prostate cancer cells (Figure S3U).

We previously reported the synergistic cell death noted with an increase in PARP cleavage following treatment with ONC201 + vorinostat or ONC201 + tazemetostat or the triple combination of ONC201 + vorinostat + tazemetostat in SU-DIPG-13, SU-DIPG-25 and SU-DIPG-29 cells, and an increase in H3K27 acetylation

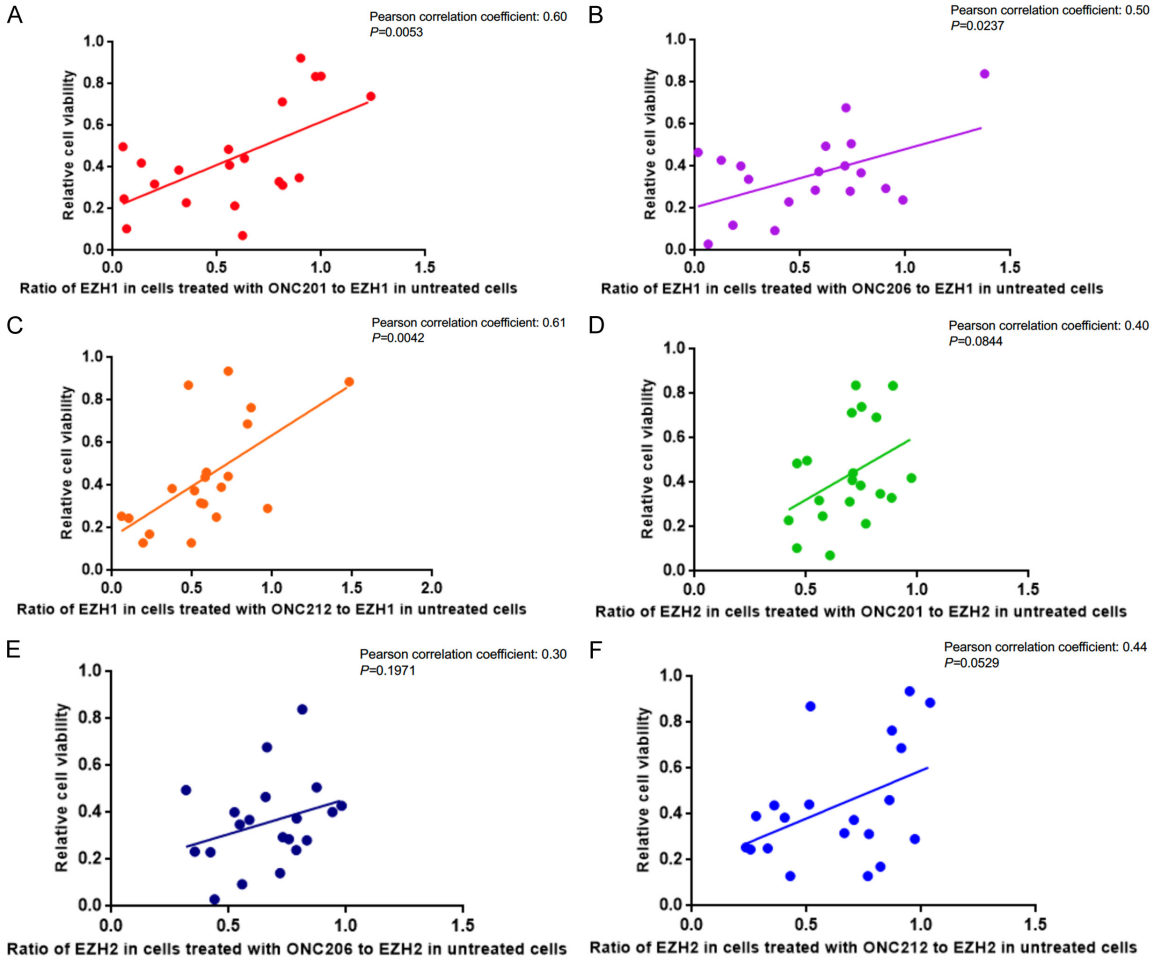


Figure 3. Cell viability suppression induced by imipridones correlates with EZH1 reduction and shows a trend to correlate with EZH2 reduction in tumor cells. Cell viability with treatment of certain doses of ONC201, ONC206 or ONC212 was obtained from dose-response relationship curves shown in Figure S2. EZH1 and EZH2 protein levels from immunoblotting shown in **Figure 1** normalized with loading control. The cell lines, doses of ONC201, ONC206 or ONC212, the normalized EZH1 or EZH2 protein level and the cell viability are displayed in Table S1.

with combinations containing vorinostat in SU-DIPG-25 and SU-DIPG-29 cells [15].

In the present study, we observed an increase in PARP cleavage in SU-DIPG-25 treated with a combination of ONC206 plus tazematostat and panobinostat and an increase in H3K27 acetylation with combinations containing Panobinostat (Figure S3T). We observed reduction of EZH1 and EZH2 by imipridones in all these cell lines (**Figures 1** and S1). Thus, H3K27 acetylation and reduction of EZH1 and EZH2 correlate with synergy when imipridones are combined with EZH inhibitors or HDAC inhibitors. SU-DIPG-4 and Hep3B are the only two cell lines in which EZH1/2 dual inhibitor valemetostat demonstrated more synergy than EHZ2 inhibitor tazemetostat when combined with ONC201 (Figure S3A and S3O).

In SU-DIPG-29 and LNCaP cells, the synergy of tazemetostat combined with ONC201 was comparable to that of valemetostat combined with ONC201 (Figure S3G and S3I). In SU-DIPG-13 and H1882 cells, synergy with tazemetostat plus ONC201 is more than that of valemetostat plus ONC201 (**Figures 3A** and S3P). Thus, it is not a universal phenomenon that dual inhibition of both EZH1 and EZH2 leads to more synergy than an EZH2-only inhibitor when they are combined with ONC201.

Imipridones share similar targets and actions in tumor cells with EZH2 inhibitors

We performed cytokine profiling assays to investigate the impact of imipridones, EHZ2i or HDACi alone or a combination of imipridones plus an EZH2 inhibitor or an HDAC inhibitor or

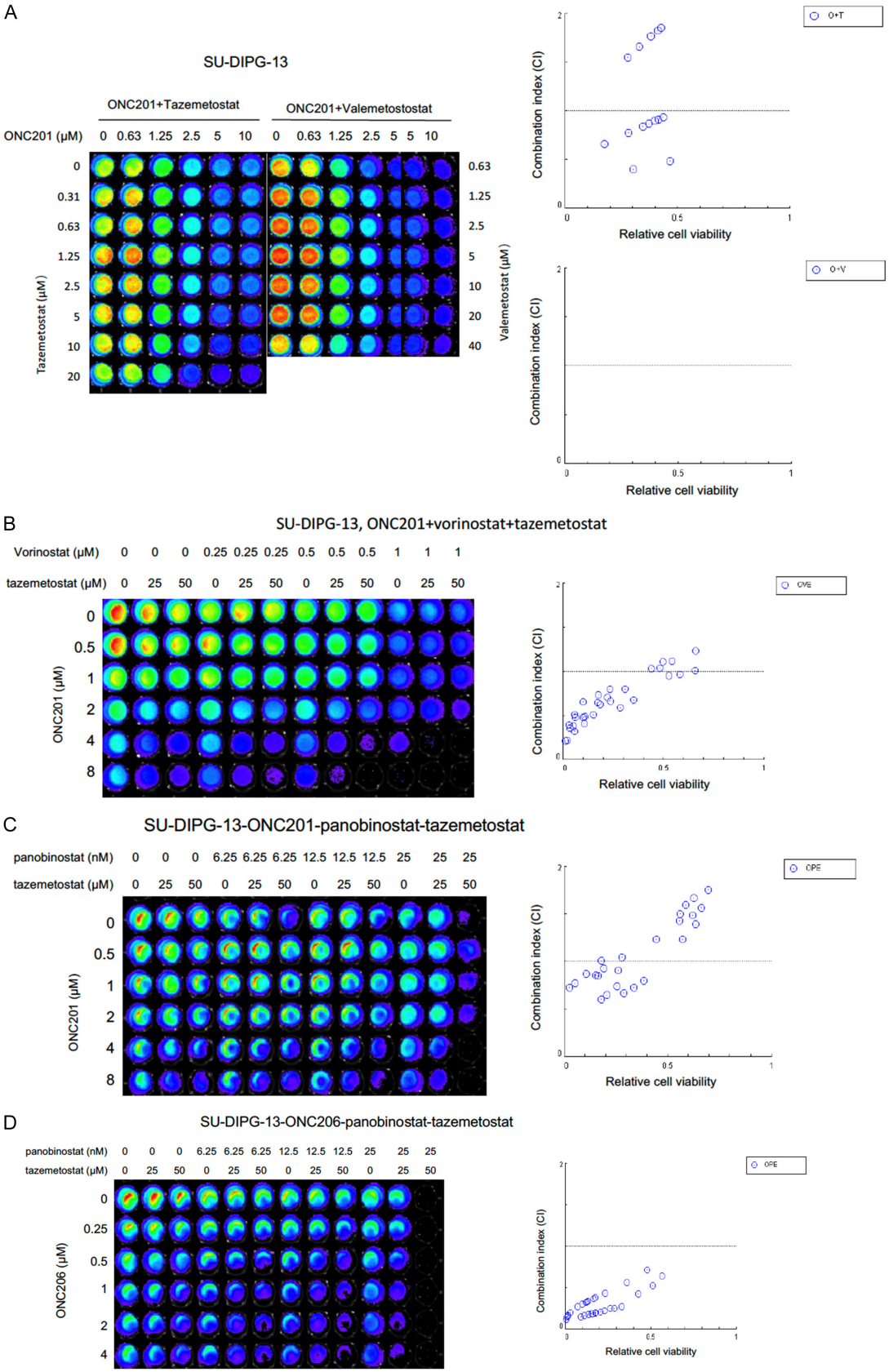
Table 1. Synergies of the combinations of imipridones with EZH inhibitors with or without HDAC inhibitors

Cell lines	Combinatorial treatment with synergies
SU-DIPG-13	ONC201 + tazemetostat (**Figure 4A**; Table S2) ONC201 + tazemetostat + vorinostat (**Figure 4B**; Table S2) ONC201 + tazemetostat + panobinostat (**Figure 4C**; Table S2) ONC206 + tazemetostat + Panobinostat (**Figure 4D**; Table S2)
SU-DIPG-4	ONC201 + tazemetostat (Figure S3A; Table S2) ONC201 + valemetostat (Figure S3A; Table S2) ONC201 + tazemetostat + vorinostat (Figure S3B; Table S2)
SU-DIPG-25	ONC201 + tazemetostat (Figure S3C; Table S2) ONC201 + tazemetostat + vorinostat (Figure S3D; Table S2) ONC201 + tazemetostat + Panobinostat (Figure S3E; Table S2) ONC206 + tazemetostat + Panobinostat (Figure S3F; Table S2)
SU-DIPG-29	ONC201 + tazemetostat (Figure S3G; Table S2) ONC201 + valemetostat (Figure S3G; Table S2)
U251	ONC201 plus valemetostat (Figure S3H; Table S2)
SNB19	ONC201 + tazemetostat (Figure S3I; Table S2) ONC201+ valemetostat (Figure S3J; Table S2) ONC206+ valemetostat (Figure S3K; Table S2)
22Rv1	ONC201+ valemetostat (**Figure 4E**; Table S2) ONC206+ valemetostat (**Figure 4F**; Table S2) ONC212+ valemetostat (**Figure 4G**; Table S2)
LNCaP	ONC201 + tazemetostat (Figure S3L; Table S2) ONC201+ valemetostat (Figure S3L; Table S2) ONC206+ valemetostat (Figure S3M; Table S2) ONC212+ valemetostat (Figure S3N; Table S2)
Hep3B	ONC201 + tazemetostat (Figure S3O; Table S2) ONC201+ valemetostat (Figure S3O; Table S2)
H1882	ONC201 + tazemetostat (Figure S3P; Table S2) ONC201+ valemetostat (Figure S3P; Table S2)
H1048	ONC201 + tazemetostat (Figure S3Q; Table S2) ONC201+ valemetostat (Figure S3R; Table S2) ONC206+ valemetostat (Figure S3S; Table S2)

the triple combination of imipridone plus EZH2 inhibitor and HDAC inhibitor on the tumor microenvironment and tumor immunity. We treated DMG, GBM and HCC cells with the drugs alone or the combinations and performed cytokine profiling assays using the cell culture supernatants. Hierarchical clustering showed that ONC201 or ONC206 induce similar cytokine profile changes with tazemetostat in T98G (**Figure 5A**) and U251 (Figure S4A) GBM, SU-DIPG-29 DMG (**Figure 5B**) and Hep3B HCC (Figure S4B) cells. Thus, based on these experiments, imipridones and tazemetostat would appear to have similar effects on the tumor microenvironment and anti-tumor immunity.

Based on our finding that imipridones reduce EZH1 and EZH2 in tumor cells, we hypothesized that imipridones have similar targets and actions with EZH1/2 inhibitors in tumor cells. We further investigated transcriptional profiles of ONC201- and tazemetostat-treated DMG cells to test the hypothesis. We treated SU-DIPG-13 and SU-DIPG-25 cells with ONC201, tazemetostat or Panobinostat and performed RNA-seq. Hierarchical clustering of the transcriptional profiles showed the similarity of ONC201 and tazemetostat treatment of SU-DIPG-13 (**Figure 5C**) and SU-DIPG-25 cells (Figure S4C).

Cluster heatmap of mRNA expression profiles of differentially expressed genes with adjusted P value < 0.05 in SU-DIPG-13 and SU-DIPG-25 cells treated with ONC201, tazemetostat or Panobinostat alone also showed similarities between ONC201 and tazemetostat treated cells (**Figures 5D**, S4D). The principal compo-


A
SU-DIPG-13
ONC201+Tazemetostat
ONC201+Valemetostostat
ONC201 (μM) 0 0.63 1.25 2.5 5 10 0 0.63 1.25 2.5 5 5 10
Tazemetostat (μM)
Valemetostat (μM)
O+T
O+V
Combination index (CI)
Relative cell viability
B
SU-DIPG-13, ONC201+vorinostat+tazemetostat
Vorinostat (μM) 0 0 0 0.25 0.25 0.25 0.5 0.5 0.5 1 1 1
tazemetostat (μM) 0 25 50 0 25 50 0 25 50 0 25 50
ONC201 (μM)
OVE
C
SU-DIPG-13-ONC201-panobinostat-tazemetostat
panobinostat (nM) 0 0 0 6.25 6.25 6.25 12.5 12.5 12.5 25 25 25
tazemetostat (μM) 0 25 50 0 25 50 0 25 50 0 25 50
ONC201 (μM)
OPE
D
SU-DIPG-13-ONC206-panobinostat-tazemetostat
panobinostat (nM) 0 0 0 6.25 6.25 6.25 12.5 12.5 12.5 25 25 25
tazemetostat (μM) 0 25 50 0 25 50 0 25 50 0 25 50
ONC206 (μM)
OPE

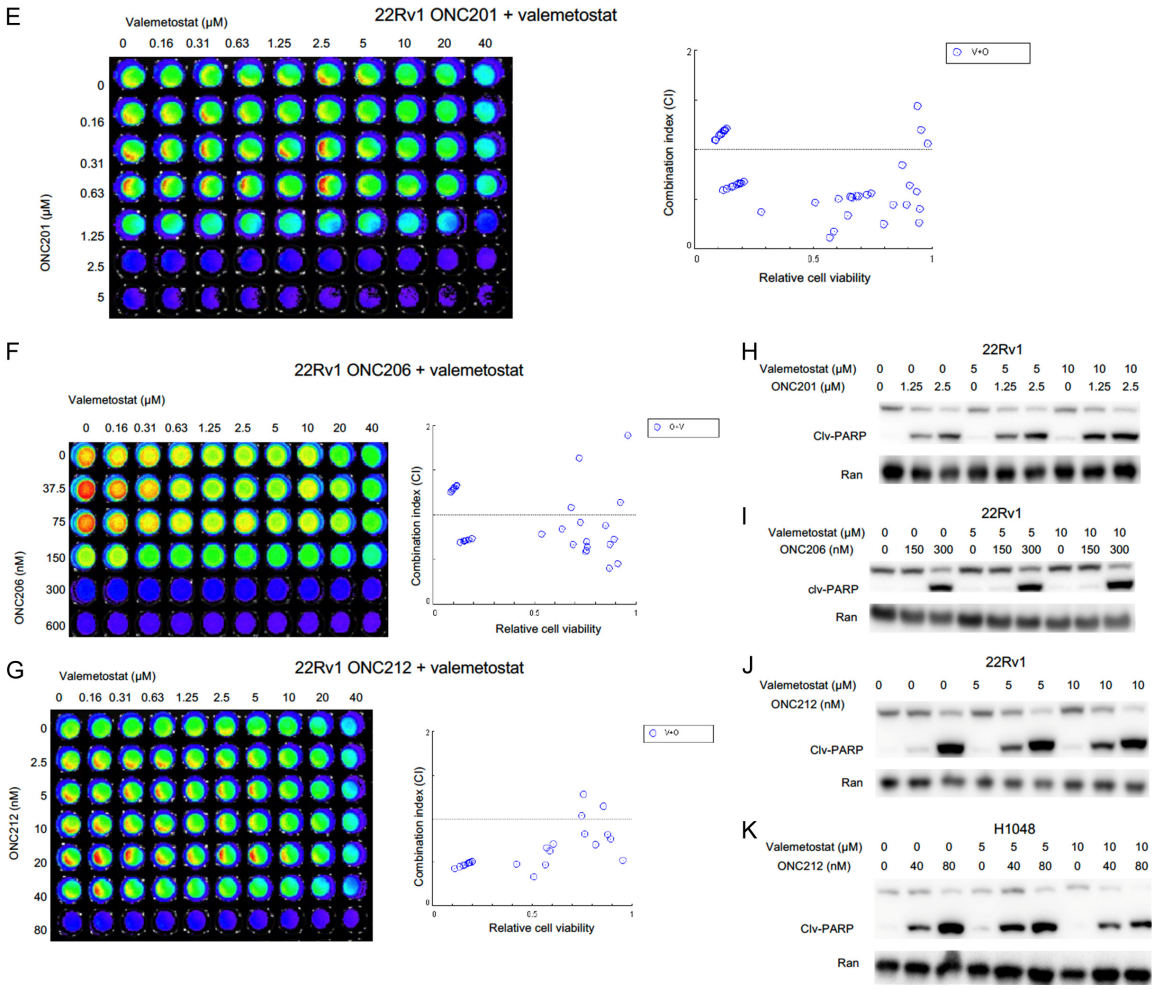


Figure 4. Combination of Imipridones with EZH inhibitors or imipridones with EZH2 inhibitor and HDAC inhibitors synergizes in suppressing cell viability and inducing apoptosis in tumor cells. Cell viability of SU-DIPG-13 cells by CellTiterGlo assay and the plots of the combination index (CI) as a function of cell viability upon 72H treatment with combination of (A) ONC201 plus tazemetostat or valemetostat, (B) ONC201 plus tazemetostat and vorinostat, (C) ONC201 plus Panobinostat and tazemetostat, (D) ONC206 plus Panobinostat and tazemetostat. Cell viability of 22Rv1 cells by CellTiterGlo assay and the plots of the CI as a function of cell viability upon 72 H treatment with combination of (E) ONC201 plus valemetostat, (F) ONC206 plus valemetostat, (G) ONC212 plus valemetostat. Immunoblotting of cleaved-PARP in 22Rv1 cells upon 48 H treatment of combination of (H) ONC201 plus valemetostat, (I) ONC206 plus valemetostat and (J) ONC212 plus valemetostat, in H1048 SCLC cells (K) treated with combination of ONC206 plus valemetostat for 48 H. Drug doses are as indicated.

nent analysis showed that the expression profiles of cells treated with ONC201 or tazemetostat were well differentiated from the control (**Figures 5E**, **5F**, S4E and S4F).

The overlaps of the top differentially expressed genes further showed the similarities of mRNA expression profile alterations between ONC201 and tazemetostat treated cells. We observed an overlap of eight genes among the top 30 differentially expressed genes sorted by adjusted *p*-value in ONC201 or tazemetostat treated SU-DIPG-13 cells (**Figure 5G**, **5H** and **Table 2**). The 8 shared genes are ALDH1B1, ARHGEF2, ATF3, ATF4, CHAC1, DDIT3, DDR2 and SARS (**Table 2**). By contrast, there was no overlap between the top 30 differentially expressed genes of cells treated with ONC201 or Panobinostat (**Figure 5G**, **5I** and **Table 2**).

There was an overlap of ten genes among the top 30 differentially expressed genes in ONC201 or tazemetostat treated SU-DIPG-25 cells (Figure S4G, S4H and Table S3). The 10 shared genes are ASNS, ATF4, DDR2, INHBE, SLC7A11, STC2, TUBE1, ULBP1, VEGFA and

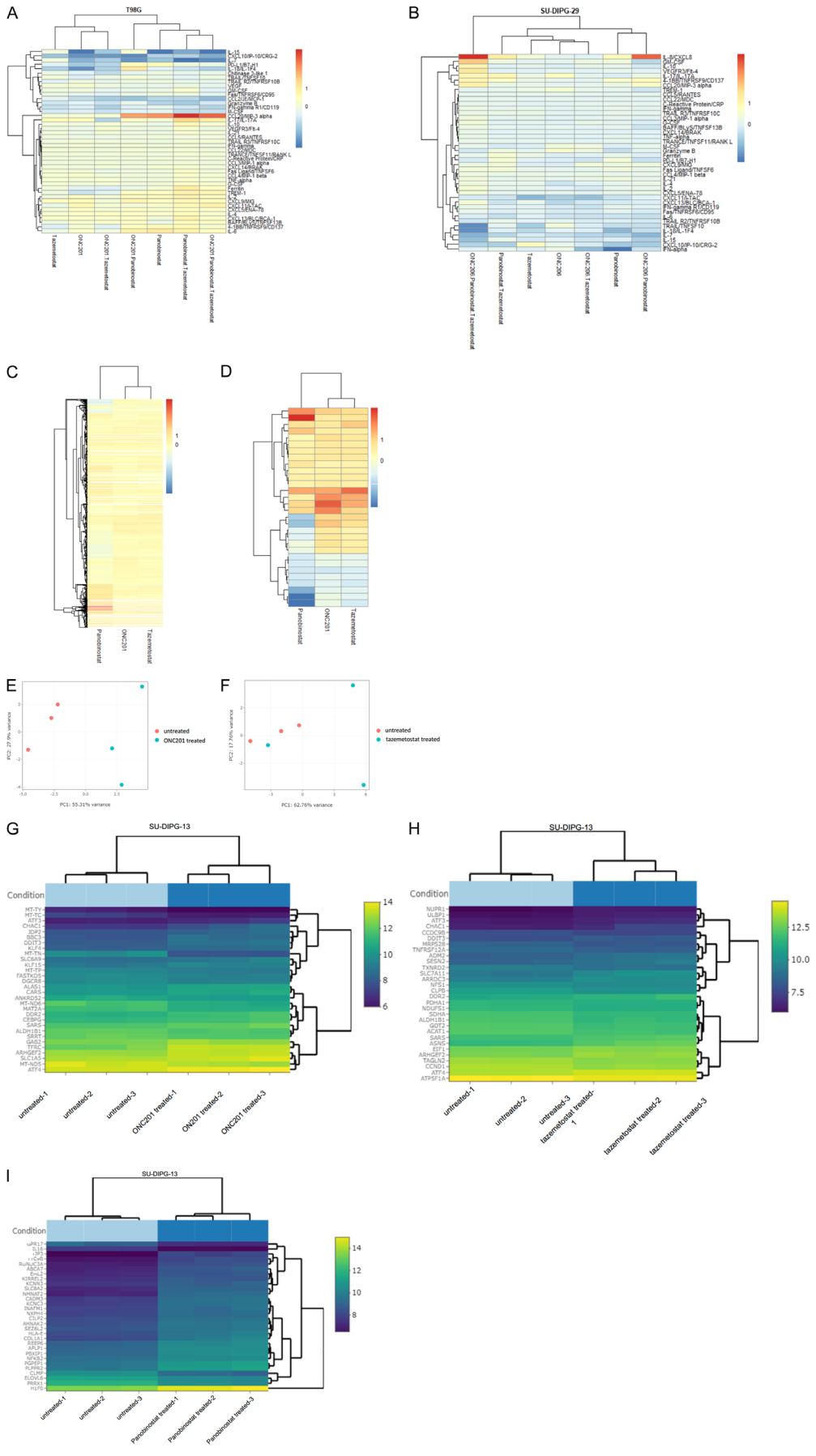

A
T98G
Tazemetostat
ONC201
ONC201 Tazemetostat
ONC201 Panobinostat
Panobinostat
Panobinostat Tazemetostat
ONC201 Panobinostat Tazemetostat
B
SU-DIPG-29
ONC206 Panobinostat Tazemetostat
Panobinostat Tazemetostat
Tazemetostat
ONC206
ONC206 Tazemetostat
Panobinostat
ONC206 Panobinostat
C
Panobinostat
ONC201
Tazemetostat
D
Panobinostat
ONC201
Tazemetostat
E
untreated
ONC201 treated
PC1: 55.31% variance
PC2: 27.... variance
F
untreated
tazemetostat treated
PC1: 62.76% variance
PC2: 17.... variance
G
SU-DIPG-13
Condition
untreated-1
untreated-2
untreated-3
ONC201 treated-1
ONC201 treated-2
ONC201 treated-3
H
SU-DIPG-13
Condition
untreated-1
untreated-2
untreated-3
tazemetostat treated-1
tazemetostat treated-2
tazemetostat treated-3
I
SU-DIPG-13
Condition
untreated-1
untreated-2
untreated-3
Panobinostat treated-1
Panobinostat treated-2
Panobinostat treated-3

Figure 5. ONC201 or ONC206 have similar effects as EZH2 inhibitor on cytokine profile and transcriptional profile in tumor cells. (A, B) Cytokine profile assay of (A) T98G GBM cells upon treatment with ONC201, tazemetostat or Panobinostat alone or the combinations indicated, and (B) SU-DIPG-29 DMG cells upon the treatment with ONC206, tazemetostat or Panobinostat alone or the combinations indicated. (C) Clustering heatmap of expression profile of SU-DIPG-13 cells treated with ONC201, tazemetostat or Panobinostat alone by plotting their log2FoldChange transformed expression values. (D) Cluster heatmap of mRNA expression profiles of differentially expressed genes with adjusted *p*-value < 0.05 in SU-DIPG-13 cells treated with ONC201, tazemetostat or Panobinostat alone by plotting their log2FoldChange transformed expression values. (E, F) The principal component analysis revealing the similarities between untreated SU-DIPG-13 cells and SU-DIPG-13 cells treated with ONC201 (E) or tazemetostat (F). (G-I) Bi-clustering heatmaps indicating the expression profile of the top 30 differentially expressed genes sorted by their adjusted *p*-value by plotting their log2 transformed expression values in SU-DIPG-13 cells treated by drugs indicated in the plot. Each group was conducted with triplicates.

Table 2. The top 30 differentially expressed genes in tazemetostat, ONC201 or Panobinostat treated SU-DIPG-13 cells

Top 30 differentially expressed genes in tazemetostat treated SU-DIPG-13 cells	Top 30 differentially expressed genes in ONC201 treated SU-DIPG-13 cells	Top 30 differentially expressed genes in panobinostat treated SU-DIPG-13 cells
ACAT1	ALAS1	ABCA7
ADM2	ALDH1B1	AHNAK2
ALDH1B1	ANKRD52	APLP1
ARHGEF2	ARHGEF2	CADM3
ARRDC3	ATF3	CILP2
ASNS	ATF4	CLMP
ATF3	BBC3	COL1A1
ATF4	CARS	ELOVL6
ATP5F1A	CEBPG	EML2
CCDC9B	CHAC1	GPR17
CCND1	DDIT3	H1F0
CHAC1	DDR2	HLA-E
CLPB	DGCR8	IL16
DDIT3	FASTKD5	INAFM1
DDR2	GAB2	KCNC3
EIF1	JDP2	KCNN3
GOT2	KLF15	KIRREL2
MRPS28	KLF4	NFKB2
NDUFS1	MAT2A	NMNAT2
NFS1	MT-ND5	NXPH4
NUPR1	MT-ND6	PBXIP1
PDHA1	MT-TC	PGPEP1
SARS	MT-TN	PLPPR2
SDHA	MT-TP	PRRX1
SESN2	MT-TY	REEP6
SLC7A11	SARS	RUNDC3A
TAGLN2	SLC1A5	SEZ6L2
TNFRSF12A	SLC6A9	SLC8A2
TXNRD2	SRRT	TJP3
ULBP1	TFRC	TTC9B

VLDLR (Table S3). There was no overlap between the top 30 differentially expressed genes of SU-DIPG-25 cells treated with ONC201 or Panobinostat (Figure S4G, S4I and Table S3).

Based on the significant overlaps of the top differentially expressed genes, we conclude that ONC201 which reduces EZH1 and EZH2 proteins share similar targets and actions with EZH2 inhibitors on tumor cells.

Discussion

TIC10/ONC201 was discovered as a TRAIL-inducing compound [1-3]. The tumoricidal effect of ONC201 and its potent analogs ONC206 and ONC212 on cancer cells was demonstrated to be related to the induction of the integrated stress response marked by upregulation of ATF4 [4] as a consequence of activation of mitochondrial caseinolytic protease ClpP [5, 6]. Clinical data showed that a subset of DMG patients benefit from ONC201 [8, 9]. Given that more than 80% of DG patients carry the driver mutation H3K27M that impairs the function of EHZ2 methylating H3K27 [10], we hypothesized that H3K27M mutation sensitization of tumors to ONC201 may be mimicked by EZH inhibitors in inhibiting methylation of H3K27. We observed synergy of imipridones plus EZH2 inhibitor, tazemetostat or dual EZH1/2 inhibitor, valemetostat in a panel of tumors from different tissue origins. Thus, inhibition of H3K27 methylation makes the tumor cells

more sensitive to imipridones as we hypothesized.

Our current results suggest that EZH1/2 may play a role in the action of imipridones and ONC201. In our studies, EZH1 and EZH2 protein expression is reduced by imipridones in all the tested tumor cell lines and the suppression of cell viability by imipridones correlates with the EZH1 and EZH2 reduction. Thus, it's reasonable to raise the question of whether ONC201 can reduce H3K27 trimethylation by decreasing EZH1 and EZH2 proteins. Our results showed that H3K27 methylation function of EZH2 was not reduced by ONC201, so the residual EZH proteins still fully conduct their histone methyltransferase function for the cells.

Cytokine profiles show similarity between ONC201 or ONC206 and tazemetostat. RNA-sequencing shows the overlap of the top differentially expressed genes in ONC201 and tazemetostat treated DMG cells whereas there was no overlap between ONC201 and Panobinostat treated cells. Therefore, ONC201 or ONC206 and EZH2 inhibitors share similar targets and actions on tumor cells, and the similarities are not dependent on the reduction of H3K27 methylation which can't be achieved by ONC201 treatment.

The findings of the action of imipridones on EZH1/2 provide new clues for elucidating the tumoricidal effect of imipridones with links to H3K27M and epigenetic alteration of gene expression and cytokine profiles.

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

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

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Imipridones suppress EZH1/2 enabling synergies with epigenetic therapies

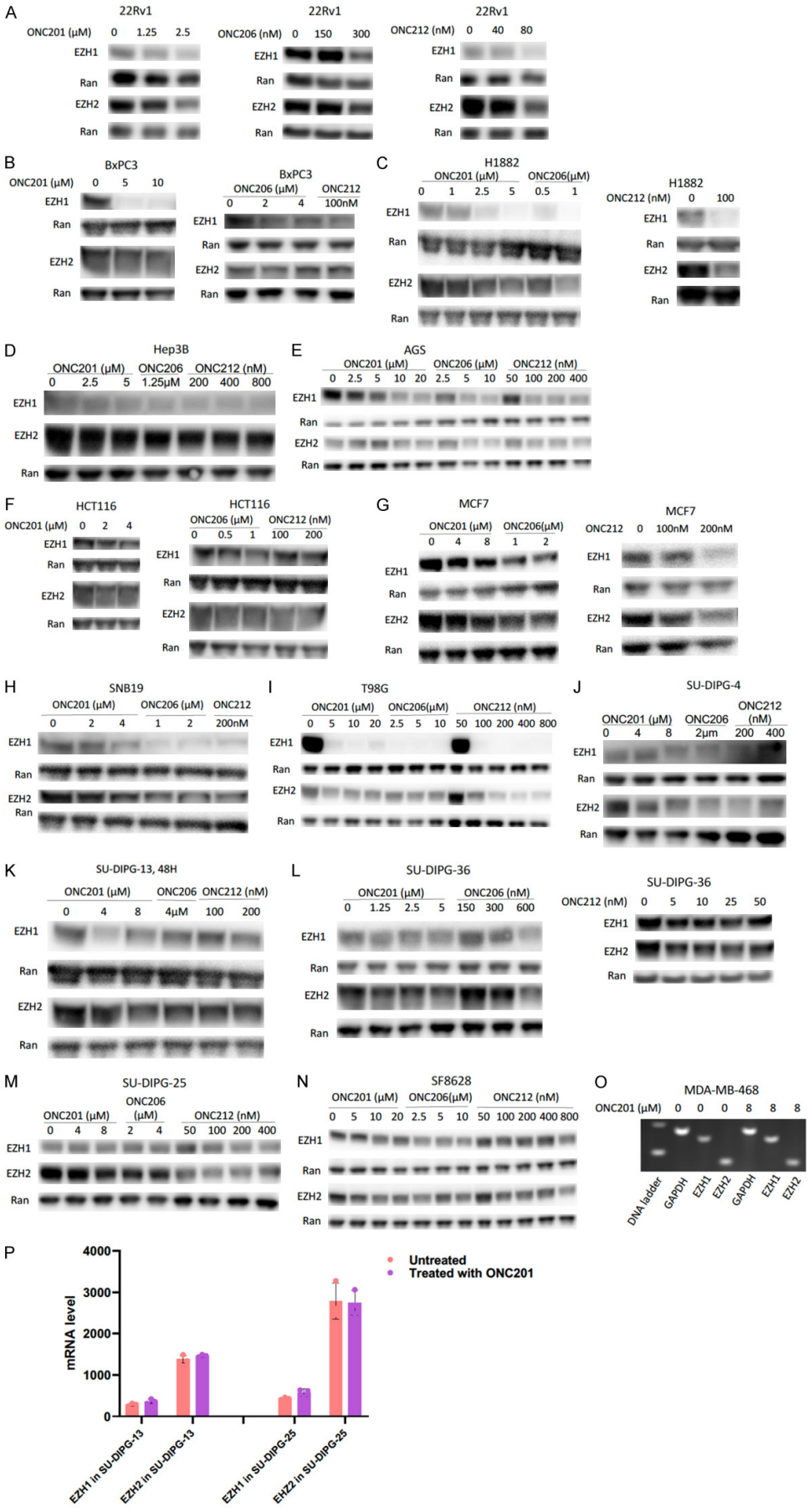
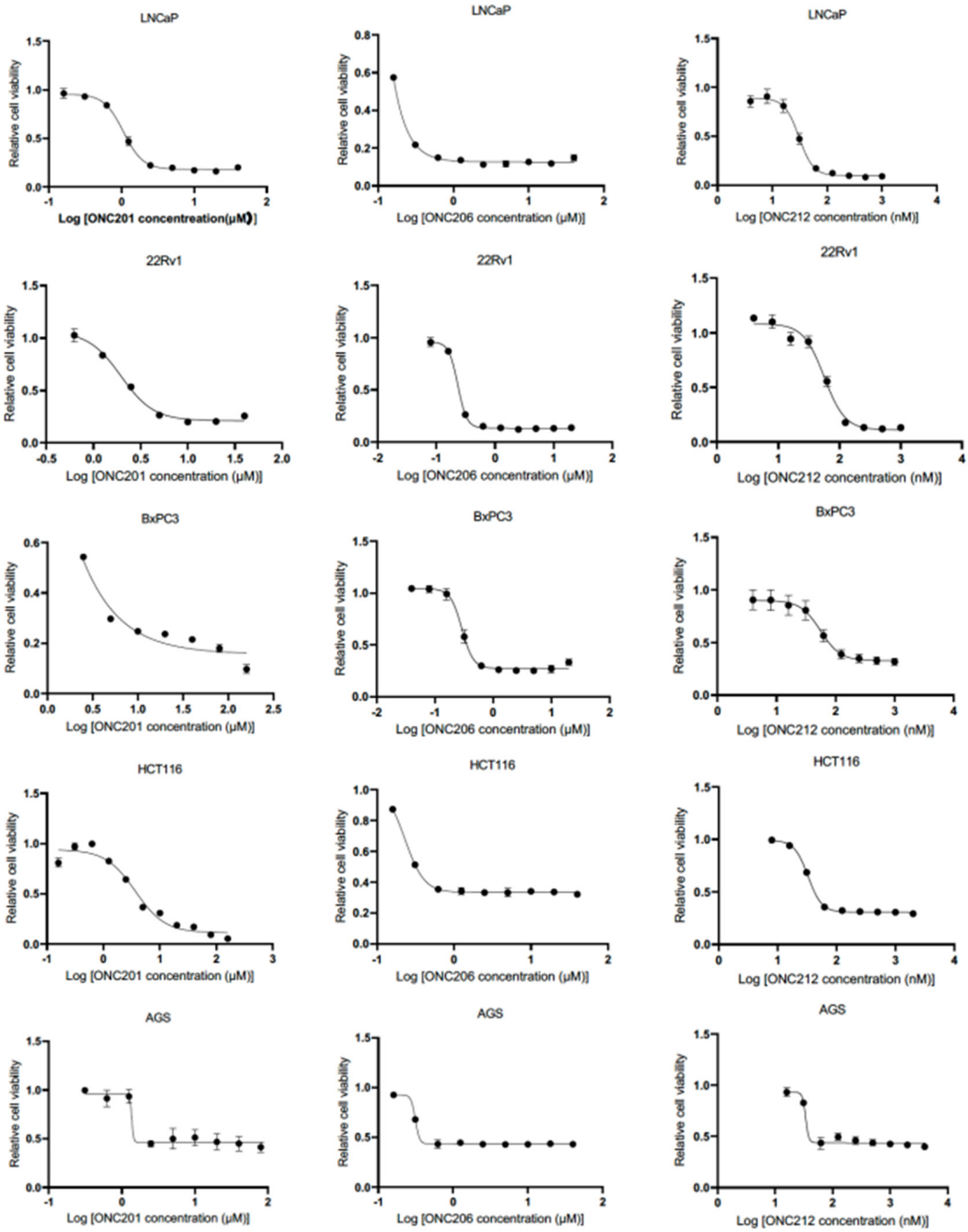
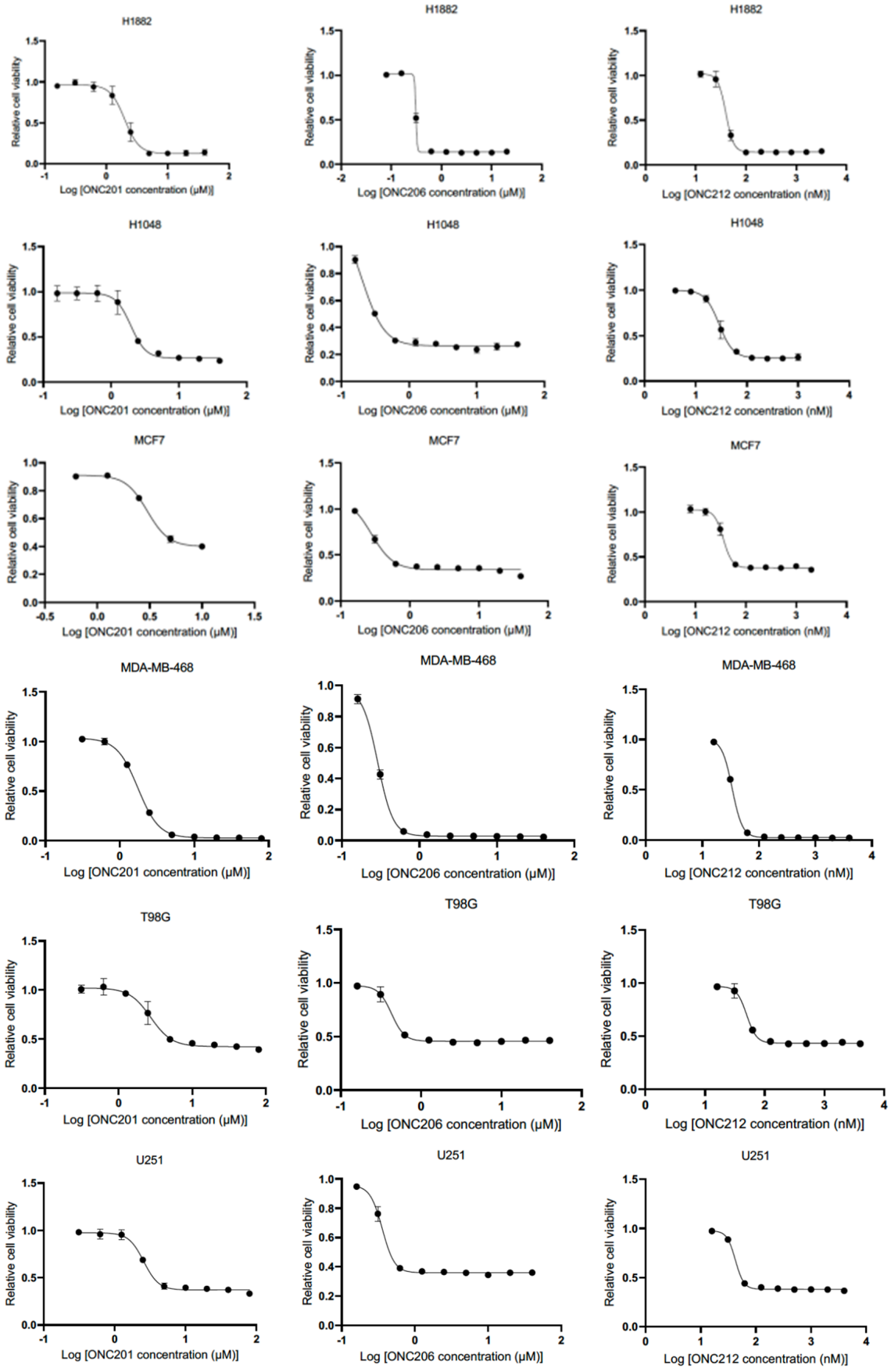


Figure S1. ONC201, ONC201 or ONC212 reduce EZH1 and EZH2 proteins in tumor cells without impacting on EZH1 and EZH2 mRNA levels. Immunoblotting of EZH1 and EZH2 in (A) 22Rv1 prostate cancer, (B) BxPC3 PDAC, (C) H1882 SCLC, (D) Hep3B HCC, (E) AGS gastric cancer, (F) HCT116 CRC, (G) MCF7 breast cancer, (H) SNB19 GBM, (I) T98G GBM, (J) SU-DIPG-4 DMG, (K) SU-DIPG-13 DMG, (L) SU-DIPG-36 DMG, (M) SU-DIPG-25 DMG, (N) SF8628 DMG. (O) mRNA level of EZH1 and EZH2 by RNA-sequencing in SU-DIPG-13 and SU- DIPG-25 cells upon treatment with ONC201 for 12 hours, (P) mRNA level of EZH1 and EZH2 by retro-transcription PCR in MDA-MB-468 cells upon treatment with ONC201 for 24 hours.




H1882
Relative cell viability
Log [ONC201 concentration (μM)]
H1882
Relative cell viability
Log [ONC206 concentration (μM)]
H1882
Relative cell viability
Log [ONC212 concentration (nM)]
H1048
Relative cell viability
Log [ONC201 concentration (μM)]
H1048
Relative cell viability
Log [ONC206 concentration (μM)]
H1048
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MCF7
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Relative cell viability
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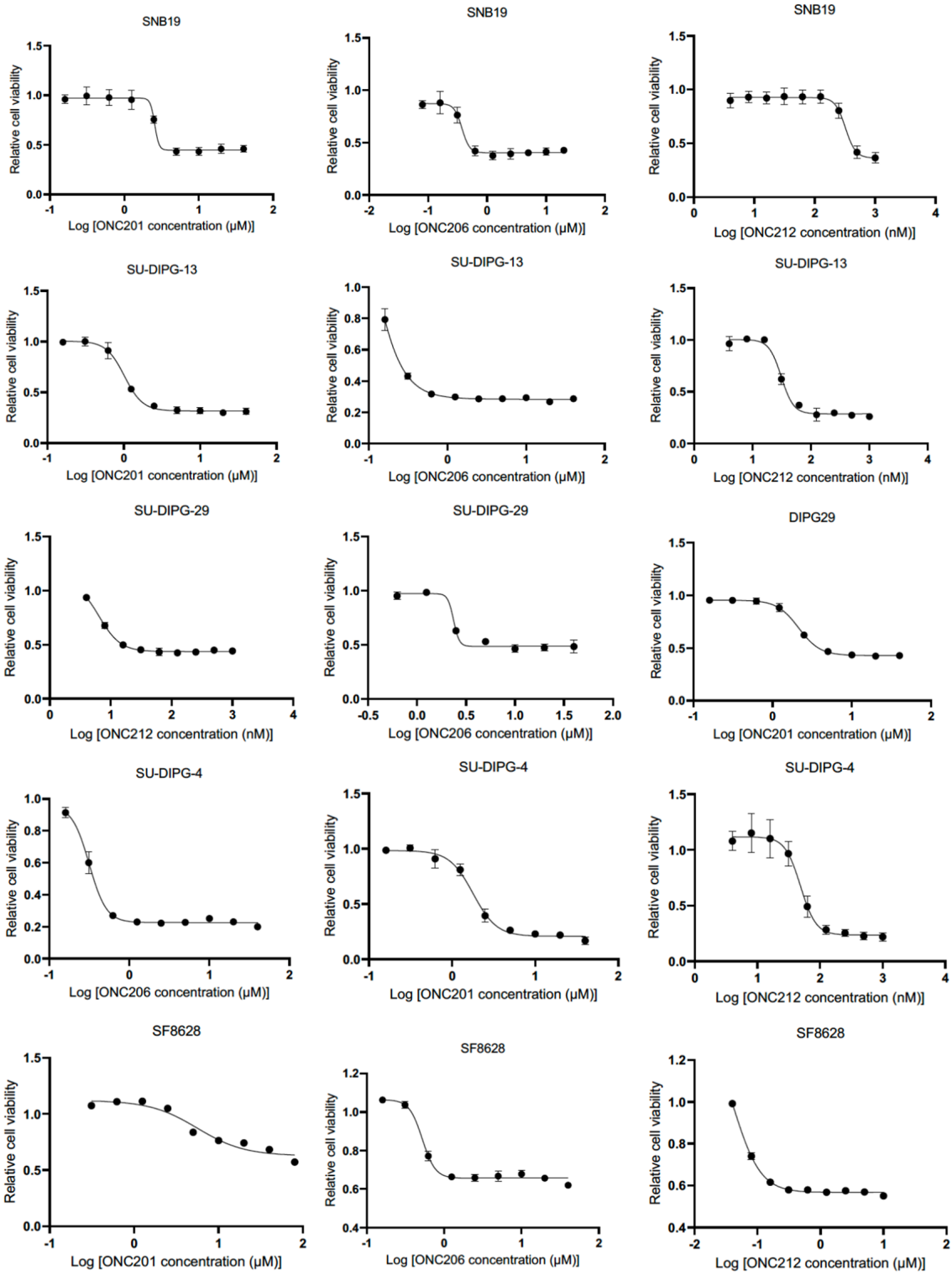
SNB19
Relative cell viability
Log [ONC201 concentration (μM)]
SNB19
Relative cell viability
Log [ONC206 concentration (μM)]
SNB19
Relative cell viability
Log [ONC212 concentration (nM)]
SU-DIPG-13
Relative cell viability
Log [ONC201 concentration (μM)]
SU-DIPG-13
Relative cell viability
Log [ONC206 concentration (μM)]
SU-DIPG-13
Relative cell viability
Log [ONC212 concentration (nM)]
SU-DIPG-29
Relative cell viability
Log [ONC212 concentration (nM)]
SU-DIPG-29
Relative cell viability
Log [ONC206 concentration (μM)]
DIPG29
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Log [ONC201 concentration (μM)]
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Relative cell viability
Log [ONC212 concentration (μM)]

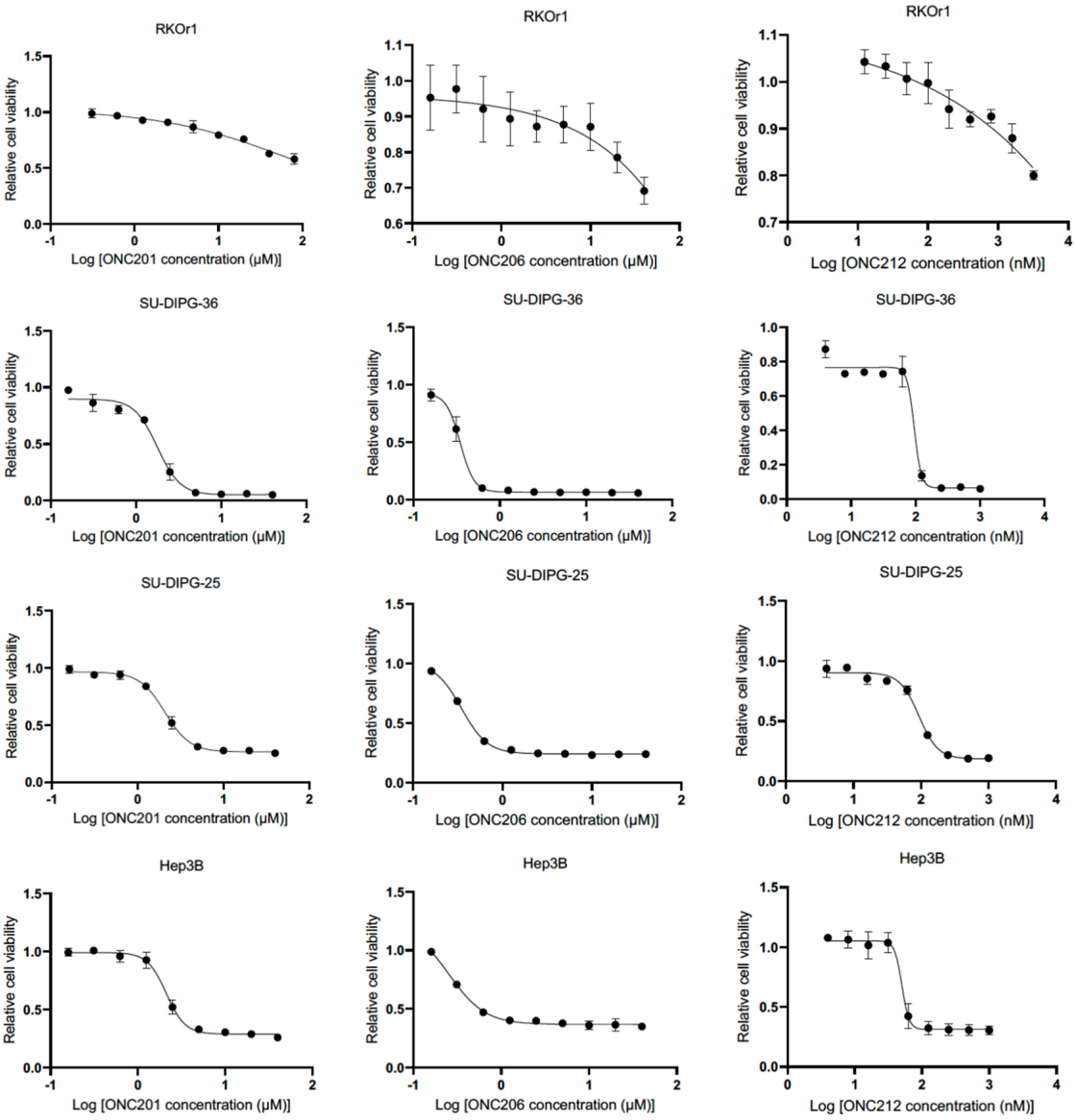
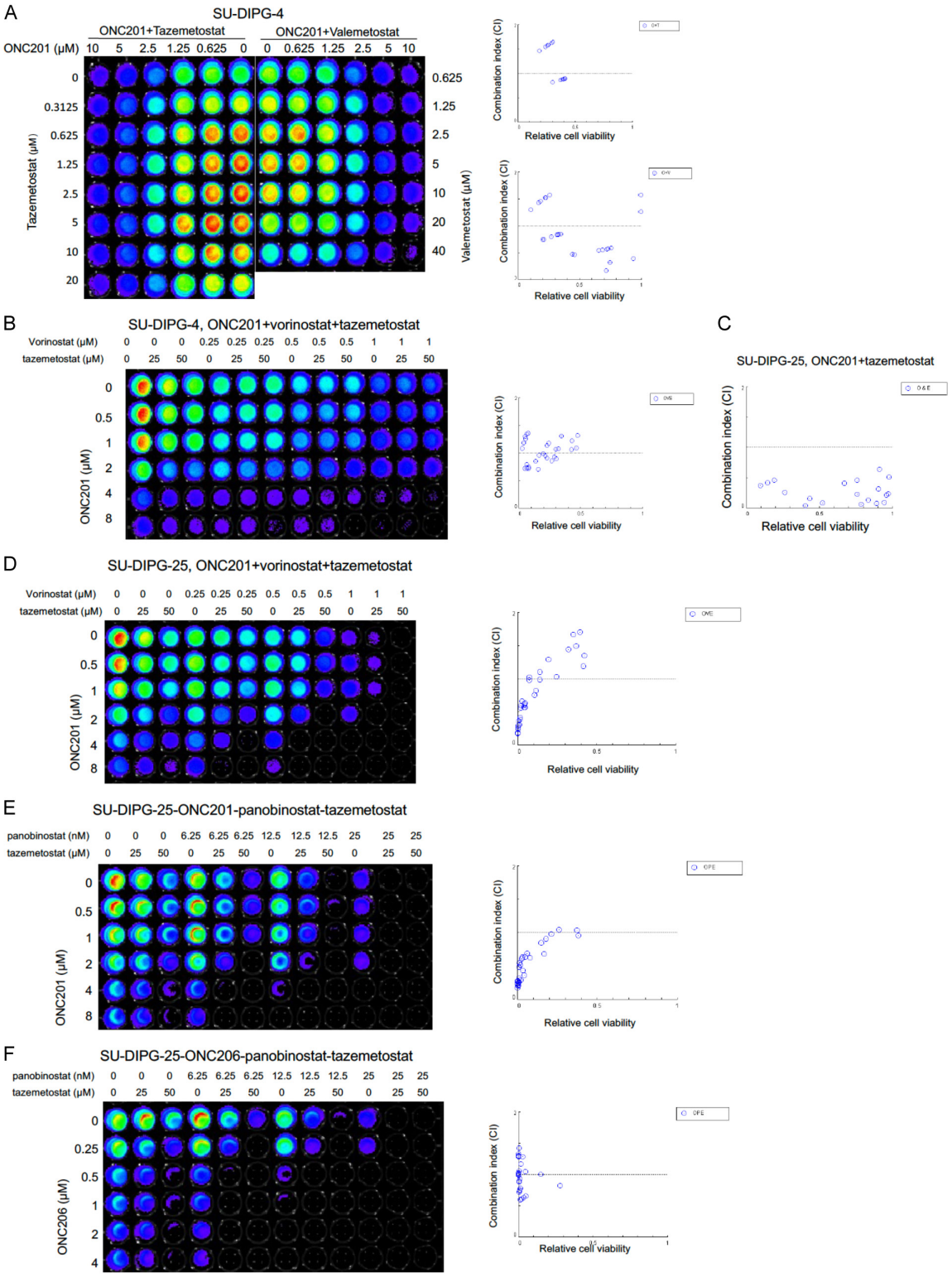
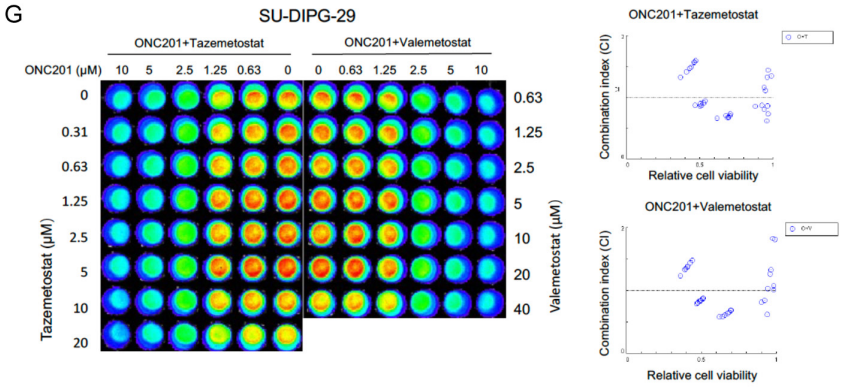


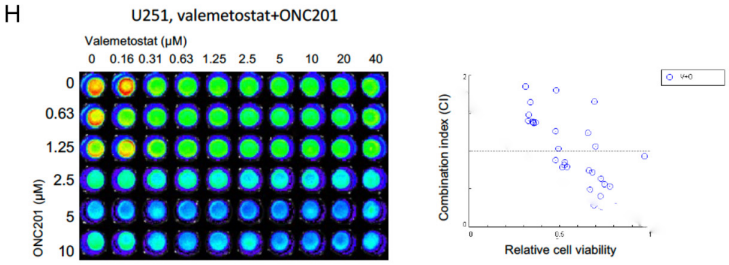
Figure S2. Dose-response relationships of ONC201, ONC206 or ONC212 on tumor cells used in **Figures 1** and S1. Cell viability was obtained by the CellTiterGlo assay after treating the indicated cell lines with ONC201, ONC206 or ONC212 for 72 hours.

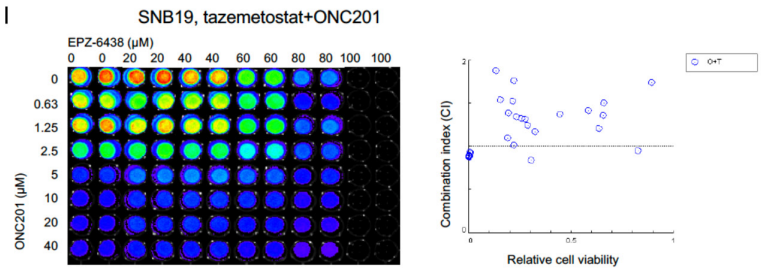
Table S1. Correlation of EZH1 protein level and cell viability

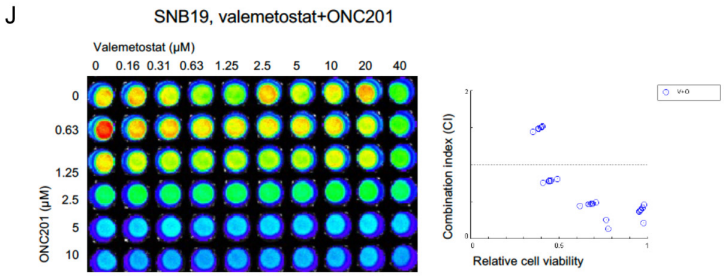
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Name of cell line	dose of ONC201 (μM	Normalized EZH1	Normalizec	cell viability			Name of cell line	dose of ONC206 (μM)	Normalizec	Normalizec	cell viability			Name of cell line	dose of ONC212 (nM)	Normalizec	Normalizec	cell viability
2	22Rv1	1.25	0.973318737	0.890693	0.8357			22Rv1	0.3	0.571728	0.755869	0.28625			22Rv1	80	0.37533	0.40456	0.385
3	AGS	20	0.137016549	0.974019	0.419277			AGS	5	0.124708	0.98091	0.428701			AGS	100	0.725109	0.510875	0.4425
4	BxPC3	10	0.053876194	0.574928	0.247883			BxPC3	2	0.738201	0.832851	0.28125			BxPC3	100	0.58914	0.861219	0.46125
5	DIPG-13	8	0.815729862	0.695849	0.3125			DIPG-13	4	0.907449	0.730151	0.295			DIPG-13	200	0.972214	0.972214	0.29125
6	DIPG-25	4	0.89492912	0.833657	0.34875			DIPG-25	4	0.989123	0.788182	0.24			DIPG-25	200	0.651313	0.330174	0.25
7	DIPG29	4	0.555217168	0.459639	0.485			DIPG29	4	0.621109	0.318155	0.495			DIPG29	200	0.581459	0.358627	0.4375
8	H1048	5	0.200164839	0.559145	0.318742			H1048	0.3	0.74277	0.874322	0.5075			H1048	200	0.058983	0.235093	0.255
9	H1882	2.5	0.317235731	0.745791	0.386493			H1882	0.5	0.180557	0.718697	0.12			H1882	100	0.194289	0.428708	0.13
10	HCT116	2	0.813609509	0.70554	0.71375			HCT116	0.5	0.588404	0.789358	0.375			HCT116	100	0.571953	0.772029	0.3125
11	LNCaP	2.5	0.352836399	0.422051	0.22875			LNCaP	0.3	0.445889	0.422122	0.23			LNCaP	80	0.495137	0.766778	0.13
12	MCF7	8	0.560531466	0.708187	0.41			MCF7	2	0.254667	0.547393	0.338			MCF7	200	0.514645	0.706141	0.375
13	MDA-MB-468	4	0.066145713	0.458901	0.10375			MDA-MB-468	1	0.062979	0.439381	0.03			MDA-MB-468	50	0.233988	0.821835	0.17
14	RKOr1	20	1.238343527	0.749404	0.74			RKOr1	10	1.376863	0.8134	0.83958			RKOr1	400	0.725998	0.949473	0.93633
15	SF8628	5	0.999904535	0.723663	0.837402			SF8628	10	0.717584	0.662788	0.677568			SF8628	50	1.48161	1.038473	0.886
16	SNB19	4	0.631677143	0.710935	0.4425			SNB19	1	0.21759	0.524254	0.40125			SNB19	200	0.477323	0.517041	0.87
17	T98G	5	0.048504706	0.504831	0.497852			T98G	2.5	0.014768	0.656797	0.466471			T98G	50	0.845758	0.91262	0.6875
18	U251	2.5	0.902409763	0.816277	0.92373			U251	1.25	0.789958	0.587717	0.369087			U251	100	0.683281	0.279903	0.39125
19	SU-DIPG-36	5	0.623087577	0.607599	0.07108			SU-DIPG-36	0.6	0.38	0.557269	0.09375			SU-DIPG-36	25	0.868155	0.871977	0.76417
20	Hep3B	5	0.797893164	0.884279	0.330785			Hep3B	1.25	0.7133	0.942346	0.40169			Hep3B	400	0.553856	0.664803	0.3175
21	SU-DIPG-4	8	0.585269214	0.768723	0.21375			SU-DIPG-4	2	0.445706	0.355604	0.2325			SU-DIPG-4	200	0.104768	0.255582	0.245

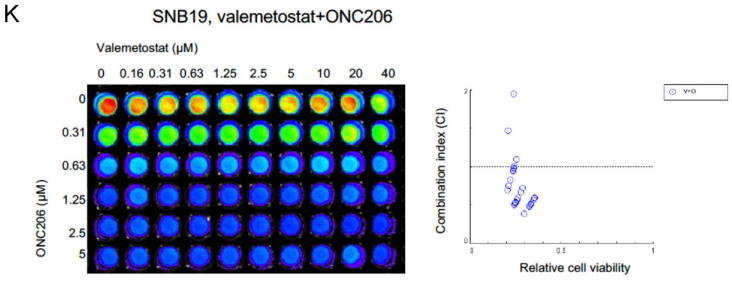

A
SU-DIPG-4
ONC201+Tazemetostat
ONC201+Valemetostat
ONC201 (μM) 10 5 2.5 1.25 0.625 0 0 0.625 1.25 2.5 5 10
Tazemetostat (μM) 0 0.3125 0.625 1.25 2.5 5 10 20
Valemetostat (μM) 0.625 1.25 2.5 5 10 20 40
Combination index (CI)
Relative cell viability
B
SU-DIPG-4, ONC201+vorinostat+tazemetostat
Vorinostat (μM) 0 0 0 0.25 0.25 0.25 0.5 0.5 0.5 1 1 1
tazemetostat (μM) 0 25 50 0 25 50 0 25 50 0 25 50
ONC201 (μM) 0 0.5 1 2 4 8
C
SU-DIPG-25, ONC201+tazemetostat
D
SU-DIPG-25, ONC201+vorinostat+tazemetostat
E
SU-DIPG-25-ONC201-panobinostat-tazemetostat
panobinostat (nM) 0 0 0 6.25 6.25 6.25 12.5 12.5 12.5 25 25 25
F
SU-DIPG-25-ONC206-panobinostat-tazemetostat
ONC206 (μM) 0 0.25 0.5 1 2 4

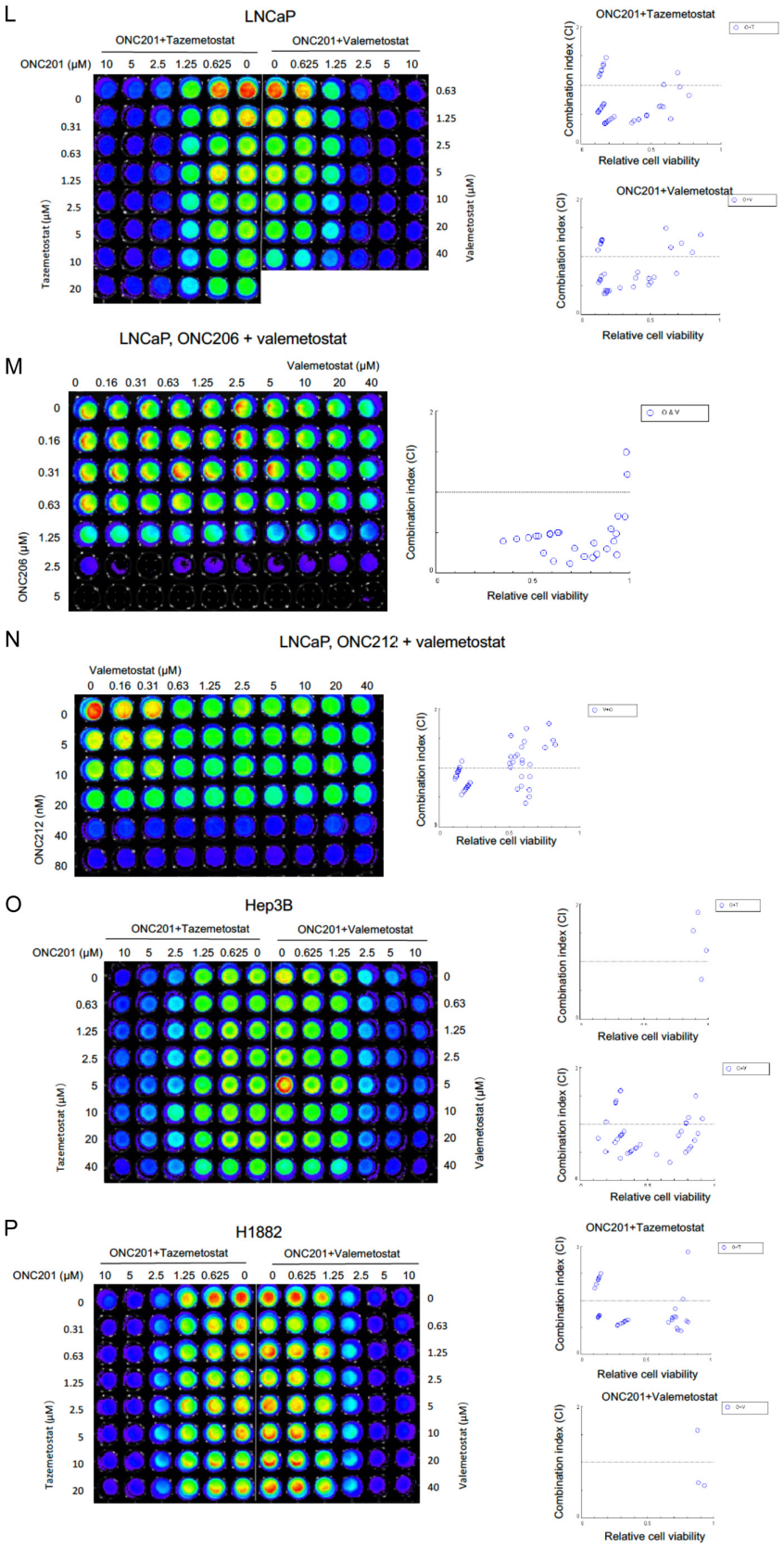

G
SU-DIPG-29
ONC201+Tazemetostat
ONC201+Valemetostat
ONC201 (μM)
Tazemetostat (μM)
Valemetostat (μM)
ONC201+Tazemetostat
ONC201+Valemetostat
Combination index (CI)
Relative cell viability


H
U251, valemetostat+ONC201
Valemetostat (μM)
ONC201 (μM)
Combination index (CI)
Relative cell viability


I
SNB19, tazemetostat+ONC201
EPZ-6438 (μM)
ONC201 (μM)
Combination index (CI)
Relative cell viability


J
SNB19, valemetostat+ONC201
Valemetostat (μM)
ONC201 (μM)
Combination index (CI)
Relative cell viability


K
SNB19, valemetostat+ONC206
Valemetostat (μM)
ONC206 (μM)
Combination index (CI)
Relative cell viability


L
LNCaP
ONC201+Tazemetostat
ONC201+Valemetostat
ONC201 (μM)
Tazemetostat (μM)
Valemetostat (μM)
ONC201+Tazemetostat
ONC201+Valemetostat
Combination index (CI)
Relative cell viability
M
LNCaP, ONC206 + valemetostat
Valemetostat (μM)
ONC206 (μM)
Combination index (CI)
Relative cell viability
N
LNCaP, ONC212 + valemetostat
Valemetostat (μM)
ONC212 (nM)
Combination index (CI)
Relative cell viability
O
Hep3B
ONC201+Tazemetostat
ONC201+Valemetostat
ONC201 (μM)
Tazemetostat (μM)
Valemetostat (μM)
Combination index (CI)
Relative cell viability
P
H1882
ONC201+Tazemetostat
ONC201+Valemetostat
ONC201 (μM)
Tazemetostat (μM)
Valemetostat (μM)
Combination index (CI)
Relative cell viability

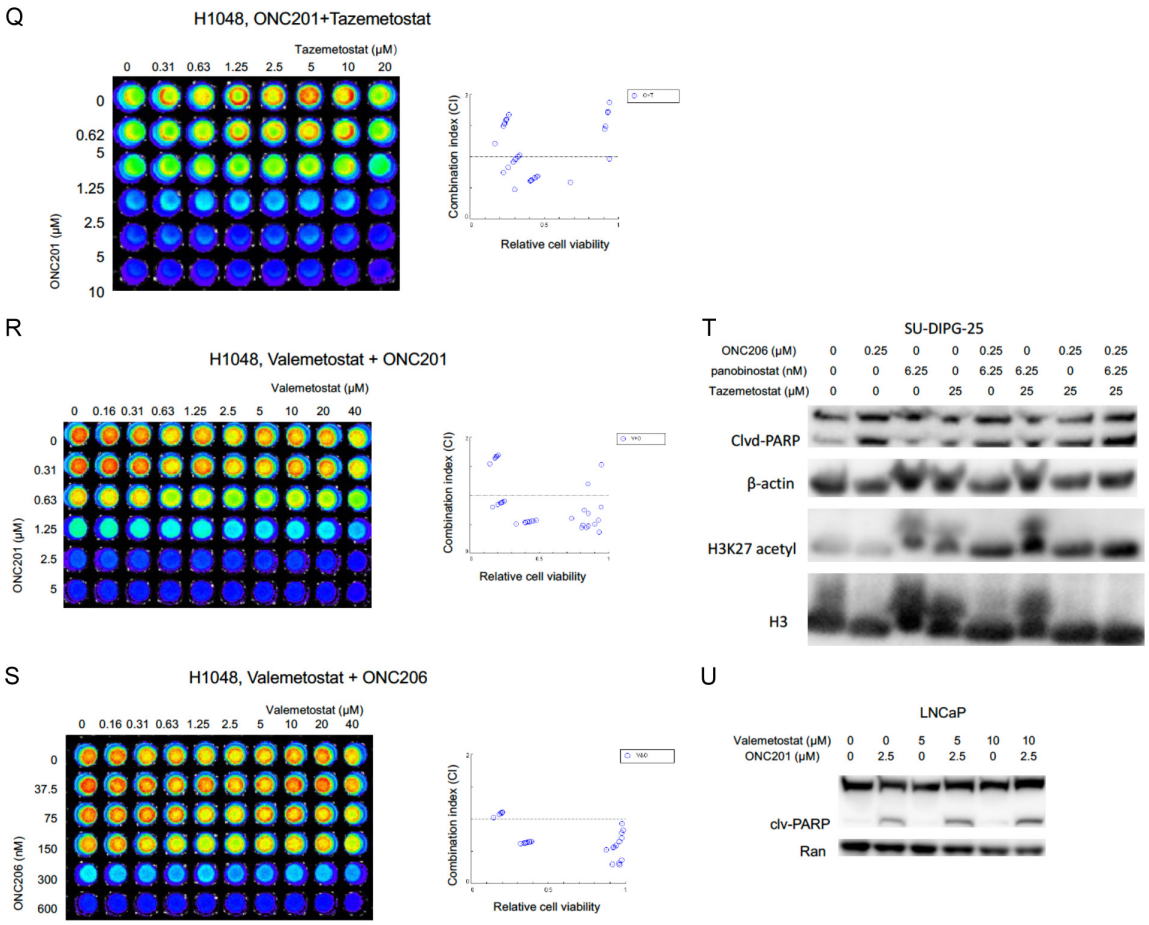


Figure S3. Combination of imipridones with EZH inhibitors or imipridones with EZH2 inhibitor and HDAC inhibitors synergize in suppressing cell viability and inducing apoptosis in tumor cells. Cell viability by CellTiterGlo assay and combination index (CI) as a function of cell viability of SU-DIPG-4 cells treated for 72H with combination of (A) ONC201 plus tazemetostat or valemetostat and (B) ONC201 plus tazemetostat and vorinostat. (C) CI as a function of cell viability of SU-DIPG-25 treated with combination of ONC201 plus tazemetostat for 72 H. Cell viability by CellTiterGlo assay and CI as a function of cell viability of SU-DIPG-25 cells treated for 72 H with combination of (D) ONC201 plus tazemetostat and vorinostat, (E) ONC201 plus tazemetostat and panobinostat, and (F) ONC206 plus tazemetostat and panobinostat. (G) Cell viability by CellTiterGlo assay and CI as a function of cell viability of SU-DIPG-29 cells treated for 72 H with combination of ONC201 plus tazemetostat or valemetostat. (H) Cell viability by CellTiterGlo assay and CI as a function of cell viability of U251 cells treated for 72 H with combination of ONC201 plus valemetostat. Cell viability by CellTiterGlo assay and CI as a function of cell viability of SNB19 cells treated for 72 H with (I) combination of ONC201 plus valemetostat ONC201 plus tazemetostat and panobinostat, (J) ONC201 plus valemetostat, (K) ONC206 plus valemetostat. Cell viability by CellTiterGlo assay and CI as a function of cell viability of LNCaP cells treated for 72 H with combination of (L) ONC201 plus tazemetostat or valemetostat, (M) ONC206 plus valemetostat, (N) ONC212 plus valemetostat. Cell viability by CellTiterGlo assay and CI as a function of cell viability of (O) Hep3B cells and (P) H1882 cells treated for 72 H with combination of ONC201 plus tazemetostat or valemetostat. Cell viability by CellTiterGlo assay and CI as a function of cell viability of H1048 cells with combination of ONC201 plus (Q) tazemetostat or (R) valemetostat, or (S) ONC206 plus valemetostat. Immunoblotting of cleaved-PARP and H3K27 acetylation of (T) SU-DIPG-25 cells treated for 48 H with combination of ONC206 plus tazemetostat and Panobinostat, and (U) LNCaP cells treated for 48 H with combination of ONC201 plus valemetostat. Drug doses are as indicated.

Table S2. CI of the combination treatments

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	SU-DIPG-4					SU-DIPG-4					SU-DIPG-4				
2	Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combinatio n Index		Dose of ONC201 (μM)	Dose of vorinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index
3	0.625	0.3125	0.99999	NaN		0.625	0.625	0.99999	1.2641		0.5	0.25	25	0.46831	1.09067
4	0.625	0.625	0.99999	NaN		0.625	1.25	0.99999	1.57909		0.5	0.25	50	0.47633	1.31492
5	0.625	1.25	0.99999	NaN		0.625	2.5	0.99999	2.20905		1	0.25	25	0.42992	1.0596
6	0.625	2.5	0.99999	NaN		0.625	5	0.99999	3.46897		1	0.25	50	0.43167	1.21861
7	0.625	5	0.99999	NaN		0.625	10	0.99999	5.98881		2	0.25	25	0.30593	0.89315
8	0.625	10	0.99999	NaN		0.625	20	0.99999	11.0285		2	0.25	50	0.26816	0.85792
9	0.625	20	0.99999	NaN		0.625	40	0.72035	0.17261		4	0.25	25	0.08158	0.7221
10	1.25	0.3125	0.99999	NaN		1.25	0.625	0.94221	0.39577		4	0.25	50	0.08305	0.7398
11	1.25	1.25	0.99999	NaN		1.25	1.25	0.99999	2.52821		8	0.25	25	0.0769	1.35545
12	1.25	2.5	0.99999	NaN		1.25	2.5	0.99999	3.15817		8	0.25	50	0.05664	1.25396
13	1.25	5	0.99999	NaN		1.25	5	0.99999	4.41809		1	0.5	25	0.32126	1.07335
14	1.25	10	0.99999	NaN		1.25	10	0.99999	6.93793		1	0.5	50	0.3475	1.30457
15	1.25	20	0.99999	NaN		1.25	20	0.99999	11.9776		1	0.5	25	0.28946	0.93104
16	2.5	0.3125	0.67907	NaN		1.25	40	0.74919	0.32459		1	0.5	50	0.30233	1.06672
17	2.5	0.625	0.78058	NaN		2.5	0.625	0.46337	0.46461		2	0.5	25	0.22844	0.9111
18	2.5	1.25	0.81035	NaN		2.5	1.25	0.72605	0.56935		2	0.5	50	0.16022	0.70865
19	2.5	2.5	0.77349	NaN		2.5	2.5	0.73349	0.57427		4	0.5	25	0.06776	0.72136
20	2.5	5	0.76116	NaN		2.5	5	0.76012	0.59139		4	0.5	50	0.06872	0.73568
21	2.5	10	0.63209	NaN		2.5	10	0.68907	0.55746		8	0.5	25	0.06644	1.34135
22	2.5	20	0.54558	NaN		2.5	20	0.6593	0.55016		8	0.5	50	0.04227	1.18279
23	5	0.3125	0.38686	0.87767		2.5	40	0.44314	0.4728		0.5	1	25	0.21723	0.95223
24	5	0.625	0.40174	0.88758		5	0.625	0.27256	0.79914		0.5	1	50	0.24439	1.17957
25	5	1.25	0.41163	0.89416		5	1.25	0.2114	0.75245		1	1	25	0.22906	1.13158
26	5	2.5	0.41291	0.89501		5	2.5	0.35105	0.85441		1	1	50	0.19901	0.99219
27	5	5	0.39419	0.88255		5	5	0.33198	0.84214		2	1	25	0.16495	0.96026
28	5	10	0.37023	0.86656		5	10	0.32477	0.83857		2	1	50	0.13892	0.84887
29	5	20	0.30535	0.82233		5	20	0.31744	0.83621		4	1	25	0.06571	0.79303
30	10	0.3125	0.24047	1.55021		5	40	0.20151	0.75161		4	1	50	0.05149	0.72137
31	10	0.625	0.26814	1.59155		10	0.625	0.16907	1.4316		8	1	25	0.05153	1.30309
32	10	1.25	0.30872	1.64936		10	1.25	0.22547	1.52719		8	1	50	0.02782	1.08474
33	10	2.5	0.26558	1.5878		10	2.5	0.25849	1.57792						
34	10	5	0.29419	1.62897		10	5	0.22372	1.5252						
35	10	10	0.25465	1.57163		10	10	0.22547	1.52898						
36	10	20	0.19035	1.46926		10	20	0.18291	1.45983						
37	1.25	0.625	0.99999	NaN		10	40	0.10397	1.29837						
38															
39															

	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
1	SU-DIPG-13					SU-DIPG-13					SU-DIPG-13				
2	Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of panobinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index
3	0.625	0.3125	0.99999	NaN		0.625	0.625	0.99999	NaN		0.5	6.25	25	0.88327	9.25804
4	0.625	0.625	0.99999	NaN		0.625	1.25	0.99999	NaN		0.5	6.25	50	0.69877	1.75674
5	0.625	1.25	0.99999	NaN		0.625	2.5	0.99999	NaN		1	6.25	25	0.77401	2.53609
6	0.625	2.5	0.99999	NaN		0.625	5	0.99999	NaN		1	6.25	50	0.62215	1.48908
7	0.625	5	0.99999	NaN		0.625	10	0.99999	NaN		2	6.25	25	0.66513	1.5713
8	0.625	10	0.99999	NaN		0.625	20	0.99999	NaN		2	6.25	50	0.58976	1.59503
9	0.625	20	0.99999	NaN		0.625	40	0.99999	NaN		4	6.25	25	0.38557	0.80098
10	1.25	0.3125	0.88689	NaN		1.25	0.625	0.68351	NaN		4	6.25	50	0.28005	1.04891
11	1.25	0.625	0.91165	NaN		1.25	1.25	0.96995	NaN		8	6.25	25	0.25501	0.74056
12	1.25	1.25	0.99999	NaN		1.25	2.5	0.96624	NaN		8	6.25	50	0.18151	1.00803
13	1.25	2.5	0.9238	NaN		1.25	5	0.99999	NaN		0.5	12.5	25	0.82683	6.71255
14	1.25	5	0.87597	NaN		1.25	10	0.92808	NaN		0.5	12.5	50	0.62913	1.67332
15	1.25	10	0.81002	NaN		1.25	20	0.94024	NaN		1	12.5	25	0.63648	1.39302
16	1.25	20	0.74418	NaN		1.25	40	0.65886	NaN		1	12.5	50	0.55812	1.42892
17	2.5	0.3125	0.57254	NaN		2.5	0.625	0.35025	Infinit		2	12.5	25	0.57394	1.23847
18	2.5	0.625	0.58436	NaN		2.5	1.25	0.49308	Infinit		2	12.5	50	0.44575	1.2357
19	2.5	1.25	0.58796	NaN		2.5	2.5	0.51109	NaN		4	12.5	25	0.33724	0.72893
20	2.5	2.5	0.56714	NaN		2.5	5	0.5363	NaN		4	12.5	50	0.19135	0.93399
21	2.5	5	0.54181	NaN		2.5	10	0.49882	Infinit		8	12.5	25	0.20855	0.6492
22	2.5	10	0.46708	0.48557		2.5	20	0.5031	NaN		8	12.5	50	0.10669	0.87646
23	2.5	20	0.30512	0.40221		2.5	40	0.44142	Infinit		0.5	25	25	0.70051	3.06688
24	5	0.3125	0.37558	0.87644		5	0.625	0.23106	Infinit		0.5	25	50	0.26274	0.91072
25	5	0.625	0.43647	0.93896		5	1.25	0.28869	Infinit		1	25	25	0.63598	2.01443
26	5	1.25	0.41666	0.91847		5	2.5	0.31435	Infinit		1	25	50	0.16494	0.84886
27	5	2.5	0.40236	0.90379		5	5	0.3247	Infinit		2	25	25	0.56347	1.50533
28	5	5	0.34575	0.84607		5	10	0.30591	Infinit		2	25	50	0.15249	0.85692
29	5	10	0.28272	0.78109		5	20	0.28205	Infinit		4	25	25	0.29015	0.67267
30	5	20	0.1776	0.66296		5	40	0.25222	Infinit		4	25	50	0.05287	0.76854
31	10	0.3125	0.42757	1.85947		10	0.625	0.16972	Infinit		8	25	25	0.18089	0.6041
32	10	0.625	0.41429	1.83206		10	1.25	0.19055	Infinit		8	25	50	0.0257	0.72727
33	10	1.25	0.41294	1.82929		10	2.5	0.21328	Infinit						
34	10	2.5	0.38379	1.76963		10	5	0.22352	Infinit						
35	10	5	0.33292	1.66595		10	10	0.20855	Infinit						
36	10	10	0.2789	1.55414		10	20	0.19966	Infinit						
37						10	40	0.15149	Infinit						
38															

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR
1		SU-DIPG-13						SU-DIPG-13					
2		Dose of ONC201 (μM)	Dose of vorinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC206 (μM)	Dose of panobinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index	
3		0.5	0.25	25	0.65943	1.00997		0.25	6.25	25	0.56928	0.63902	
4		0.5	0.25	50	0.66095	1.23849		0.25	6.25	50	0.48143	0.71262	
5		1	0.25	25	0.58426	0.96452		0.5	6.25	25	0.33131	0.27155	
6		1	0.25	50	0.53027	0.95085		0.5	6.25	50	0.23603	0.42949	
7		2	0.25	25	0.35156	0.67597		1	6.25	25	0.23072	0.21751	
8		2	0.25	50	0.28502	0.59192		1	6.25	50	0.16675	0.36841	
9		4	0.25	25	0.15333	0.51233		2	6.25	25	0.20717	0.20807	
10		4	0.25	50	0.10888	0.40944		2	6.25	50	0.13536	0.33871	
11		8	0.25	25	0.10091	0.65902		4	6.25	25	0.18143	0.19711	
12		8	0.25	50	0.06261	0.48052		4	6.25	50	0.12194	0.32551	
13		0.5	0.5	25	0.5449	1.11837		0.25	12.5	25	0.51451	0.52615	
14		0.5	0.5	50	0.48355	1.04077		0.25	12.5	50	0.36363	0.56048	
15		1	0.5	25	0.49743	1.11106		0.5	12.5	25	0.28886	0.25264	
16		1	0.5	50	0.43996	1.03072		0.5	12.5	50	0.17873	0.38073	
17		2	0.5	25	0.30928	0.80103		1	12.5	25	0.17671	0.19226	
18		2	0.5	50	0.23835	0.66092		1	12.5	50	0.12928	0.33304	
19		4	0.5	25	0.11155	0.48877		2	12.5	25	0.16186	0.18549	
20		4	0.5	50	0.06014	0.32037		2	12.5	50	0.10169	0.30346	
21		8	0.5	25	0.05908	0.50771		4	12.5	25	0.13932	0.17431	
22		8	0.5	50	0.03437	0.35633		4	12.5	50	0.07451	0.27025	
23		0.5	1	25	0.22296	0.70412		0.25	25	25	0.42928	0.42138	
24		0.5	1	50	0.18577	0.62367		0.25	25	50	0.00613	0.11408	
25		1	1	25	0.2337	0.80127		0.5	25	25	0.26456	0.2491	
26		1	1	50	0.17518	0.64514		0.5	25	50	0.03065	0.19729	
27		2	1	25	0.17679	0.73315		1	25	25	0.15662	0.18461	
28		2	1	50	0.1005	0.48025		1	25	50	0.01617	0.15839	
29		4	1	25	0.05197	0.37885		2	25	25	0.11409	0.16072	
30		4	1	50	0.02357	0.22192		2	25	50	0.01775	0.1635	
31		8	1	25	0.03108	0.39556		4	25	25	0.09257	0.14784	
32		8	1	50	0.01234	0.21555		4	25	50	0.01171	0.14195	
33													

	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF
1	SU-DIPG-29					SU-DIPG-29					SU-DIPG-25			
2	Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index
3	0.625	0.3125	0.95928	0.62291		0.625	0.625	0.98255	1.00834		0.125	5	0.94283	0.0936
4	0.625	0.625	0.96917	0.73847		0.625	1.25	0.98429	1.07609		0.125	10	0.89215	0.07612
5	0.625	1.25	0.99999	66.6479		0.625	2.5	0.9936	1.81179		0.125	20	0.78605	0.06052
6	0.625	2.5	0.98837	1.35092		0.625	5	0.99999	78.6693		0.125	40	0.40589	0.03813
7	0.625	5	0.99999	70.5212		0.625	10	1	394.94		0.25	5	0.97006	0.23566
8	0.625	10	0.96393	0.8662		0.625	20	0.99999	118.607		0.25	10	0.9581	0.21189
9	0.625	20	0.94008	0.80858		0.625	40	0.94124	0.61924		0.25	20	0.83348	0.13173
10	1.25	0.3125	0.92612	0.87203		1.25	0.625	0.92205	0.84142		0.25	40	0.52198	0.08667
11	1.25	0.625	0.95055	1.11236		1.25	1.25	0.96393	1.32951		0.5	5	0.97769	0.51375
12	1.25	1.25	0.968	1.44583		1.25	2.5	0.94415	1.03317		0.5	10	0.90407	0.31334
13	1.25	2.5	0.96161	1.32624		1.25	5	0.99244	3.29592		0.5	20	0.75797	0.2312
14	1.25	5	0.99999	135.878		1.25	10	0.9587	1.26425		0.5	40	0.43363	0.15731
15	1.25	10	0.94183	1.1616		1.25	20	0.97673	1.83175		1	5	0.91121	0.63981
16	1.25	20	0.879	0.85932		1.25	40	0.90285	0.81398		1	10	0.75994	0.4636
17	2.5	0.3125	0.69284	0.66809		2.5	0.625	0.64456	0.59008		1	20	0.67268	0.412
18	2.5	0.625	0.69866	0.6804		2.5	1.25	0.68586	0.65493		1	40	0.26394	0.25576
19	2.5	1.25	0.69866	0.68438		2.5	2.5	0.65852	0.6118		2	5	0.19217	0.45729
20	2.5	2.5	0.70797	0.70974		2.5	5	0.69983	0.68194		2	10	0.14656	0.41837
21	2.5	5	0.71146	0.73285		2.5	10	0.67597	0.64398		2	20	0.09885	0.37018
22	2.5	10	0.68179	0.70859		2.5	20	0.69983	0.69219		2	40	0.09885	0.37018
23	2.5	20	0.62071	0.66473		2.5	40	0.6242	0.58255					
24	5	0.3125	0.50588	0.85856		5	0.625	0.46981	0.79102					
25	5	0.625	0.51251	0.87273		5	1.25	0.49575	0.83849					
26	5	1.25	0.50384	0.85869		5	2.5	0.4772	0.80483					
27	5	2.5	0.52728	0.91046		5	5	0.51082	0.86856					
28	5	5	0.53752	0.94302		5	10	0.49901	0.84771					
29	5	10	0.50657	0.90172		5	20	0.51291	0.87818					
30	5	20	0.47184	0.87524		5	40	0.48965	0.84044					
31	10	0.3125	0.47394	1.5976		10	0.625	0.36312	1.23669					
32	10	0.625	0.44543	1.49965		10	1.25	0.41129	1.38541					
33	10	1.25	0.46603	1.57328		10	2.5	0.39994	1.34981					
34	10	2.5	0.43537	1.47292		10	5	0.44084	1.48324					
35	10	5	0.46137	1.57178		10	10	0.40727	1.37495					
36	10	10	0.40925	1.41437		10	20	0.42897	1.44804					
37	10	20	0.37097	1.32639		10	40	0.3929	1.33732					
38														
39														

	BH	BI	BJ	BK	BL	BM	BN	BO	BP	BQ	BR	BS	BT	BU	BV	BW	BX
1	SU-DIPG-25						SU-DIPG-25						SU-DIPG-25				
2	Dose of ONC201 (μM)	Dose of panobinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of vorinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC206 (μM)	Dose of panobinostat (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index
3	0.5	6.25	25	0.38262	0.96293		0.5	0.25	25	0.41673	1.19231		0.25	6.25	25	0.27925	0.82607
4	0.5	6.25	50	0.18129	0.90991		0.5	0.25	50	0.37032	1.49323		0.25	6.25	50	0.04838	1.04786
5	1	6.25	25	0.37295	1.0398		1	0.25	25	0.42337	1.35013		0.5	6.25	25	0.05088	0.65494
6	1	6.25	50	0.14863	0.84962		1	0.25	50	0.32278	1.4441		0.5	6.25	50	0.01039	0.93073
7	2	6.25	25	0.16889	0.68532		2	0.25	25	0.24595	1.03428		1	6.25	25	0.03476	0.63717
8	2	6.25	50	0.03586	0.4389		2	0.25	50	0.11673	0.82173		1	6.25	50	0.01073	0.9344
9	4	6.25	25	0.04427	0.36711		4	0.25	25	0.10797	0.75906		2	6.25	25	0.02156	0.6142
10	4	6.25	50	0.00893	0.24086		4	0.25	50	0.04668	0.56856		2	6.25	50	0.0057	0.89203
11	8	6.25	25	0.02207	0.29384		8	0.25	25	0.04749	0.63117		4	6.25	25	0.01469	0.59898
12	8	6.25	50	0.00653	0.22089		8	0.25	50	0.01604	0.3903		4	6.25	50	0.00476	0.88192
13	0.5	12.5	25	0.26125	1.04802		0.5	0.5	25	0.39509	1.70712		0.25	12.5	25	0.14932	1.00697
14	0.5	12.5	50	0.06097	0.68886		0.5	0.5	50	0.19449	1.29177		0.25	12.5	50	0.01561	1.1699
15	1	12.5	25	0.21395	0.98195		1	0.5	25	0.35219	1.66851		0.5	12.5	25	0.01243	0.78149
16	1	12.5	50	0.05039	0.64281		1	0.5	50	0.13977	1.10579		0.5	12.5	50	0.003	1.02578
17	2	12.5	25	0.08038	0.62588		2	0.5	25	0.13977	0.98834		1	12.5	25	0.0082	0.754
18	2	12.5	50	0.01008	0.32314		2	0.5	50	0.04296	0.61128		1	12.5	50	0.00236	1.00693
19	4	12.5	25	0.01116	0.26454		4	0.5	25	0.04136	0.56754		2	12.5	25	0.00658	0.74078
20	4	12.5	50	0.0033	0.20379		4	0.5	50	0.00977	0.30612		2	12.5	50	0.00226	1.00359
21	8	12.5	25	0.00614	0.21466		8	0.5	25	0.01623	0.41723		4	12.5	25	0.00493	0.72371
22	8	12.5	50	0.00224	0.17716		8	0.5	50	0.0048	0.24405		4	12.5	50	0.00207	0.99744
23	0.5	25	25	0.03285	0.63573		0.5	1	25	0.07331	0.98319		0.25	25	25	0.0274	1.2877
24	0.5	25	50	0.01425	0.5436		0.5	1	50	0.02172	0.59914		0.25	25	50	0.00445	1.42641
25	1	25	25	0.02907	0.6103		1	1	25	0.07463	1.02349		0.5	25	25	0.00339	1.0548
26	1	25	50	0.01071	0.48561		1	1	50	0.01863	0.56513		0.5	25	50	0.00194	1.32831
27	2	25	25	0.01893	0.51865		2	1	25	0.02989	0.66012		1	25	25	0.00246	1.02359
28	2	25	50	0.00275	0.27948		2	1	50	0.00478	0.29145		1	25	50	0.00156	1.30376
29	4	25	25	0.00408	0.27925		4	1	25	0.00851	0.369		2	25	25	0.0017	0.98932
30	4	25	50	0.00191	0.24269		4	1	50	0.00175	0.18344		2	25	50	0.00138	1.29032
31	8	25	25	0.00257	0.23624		8	1	25	0.00367	0.26644		4	25	25	0.00222	1.0152
32	8	25	50	0.00244	0.27344		8	1	50	0.00139	0.17769		4	25	50	0.00136	1.28858
33																	
34																	

	BZ	CA	CB	CC	CD	CE	CF	CG	CH	CI	CJ	CK	CL	CM	CN	CO	CP	CQ	CR
1	LNCaP					LNCaP					LNCaP					LNCaP			
2	Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC206 (nM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC212 (nM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index
3	0.625	0.3125	0.77205	0.82937		0.625	0.625	0.86606	1.38129		19	0.16	0.98911	1.22259		5	0.16	0.83021	1.39609
4	0.625	0.625	0.64116	0.43315		1.25	0.625	0.48821	0.52091		37.5	0.16	0.99999	1.15E+07		10	0.16	0.75693	1.34546
5	0.625	1.25	0.84658	6.19526		2.5	0.625	0.20846	0.42016		75	0.16	0.97774	0.6961		20	0.16	0.58847	1.34851
6	0.625	2.5	0.70428	0.97229		5	0.625	0.17034	0.70679		150	0.16	0.63112	0.50241		40	0.16	0.20795	0.69723
7	0.625	5	0.68907	1.21711		10	0.625	0.152	1.28679		19	0.3125	0.99999	2.25E+07		80	0.16	0.13811	0.94149
8	0.625	10	0.58669	0.63368		0.625	1.25	0.80323	1.07369		37.5	0.3125	0.93456	0.22503		5	0.3125	0.81367	1.46959
9	0.625	20	0.59363	1.00781		1.25	1.25	0.50304	0.55964		75	0.3125	0.92038	0.39257		10	0.3125	0.78463	1.75234
10	1.25	0.3125	0.57215	0.64616		2.5	1.25	0.18755	0.38479		150	0.3125	0.59149	0.4852		20	0.3125	0.60769	1.44621
11	1.25	0.625	0.46673	0.4806		5	1.25	0.13897	0.59961		19	0.625	0.98239	1.49332		40	0.3125	0.19727	0.66152
12	1.25	1.25	0.5577	0.63776		10	1.25	0.15361	1.29862		37.5	0.625	0.99999	4.50E+07		80	0.3125	0.13834	0.9429
13	1.25	2.5	0.47044	0.49588		0.625	2.5	0.69011	0.71559		75	0.625	0.99364	15.3583		5	0.625	0.61573	0.40241
14	1.25	5	0.41426	0.42425		1.25	2.5	0.53023	0.64651		150	0.625	0.59344	0.48617		10	0.625	0.58668	0.68993
15	1.25	10	0.36473	0.36848		2.5	2.5	0.17671	0.36789		19	1.25	0.99999	8.99E+07		20	0.625	0.52458	1.10144
16	1.25	20	0.37605	0.39591		5	2.5	0.12776	0.56186		37.5	1.25	0.98116	2.58146		40	0.625	0.18038	0.60594
17	2.5	0.3125	0.23783	0.47044		10	2.5	0.1442	1.23528		75	1.25	0.93126	0.49077		80	0.625	0.12525	0.86081
18	2.5	0.625	0.1712	0.35441		0.625	5	0.727	1.2344		150	1.25	0.63894	0.5068		5	1.25	0.64388	0.51268
19	2.5	1.25	0.22072	0.44025		1.25	5	0.49049	0.62719		19	2.5	0.99999	1.80E+08		10	1.25	0.56032	0.64309
20	2.5	2.5	0.19049	0.38768		2.5	5	0.19202	0.39988		37.5	2.5	0.99999	1.80E+08		20	1.25	0.54513	1.19106
21	2.5	5	0.17947	0.36875		5	5	0.13992	0.60658		75	2.5	0.94154	0.71009		40	1.25	0.18999	0.63747
22	2.5	10	0.17253	0.35695		10	5	0.15	1.27808		150	2.5	0.53266	0.46203		80	1.25	0.12341	0.84937
23	2.5	20	0.20523	0.41427		0.625	10	0.65067	1.16406		19	5	0.9737	4.34656		5	2.5	0.64477	0.62517
24	5	0.3125	0.16217	0.67786		1.25	10	0.38156	0.48183		37.5	5	0.99999	3.60E+08		10	2.5	0.6479	1.05094
25	5	0.625	0.13251	0.57619		2.5	10	0.16797	0.36334		75	5	0.90533	0.5451		20	2.5	0.62422	1.67184
26	5	1.25	0.15637	0.65802		5	10	0.13679	0.60029		150	5	0.47909	0.44191		40	2.5	0.20304	0.6809
27	5	2.5	0.14135	0.60658		10	10	0.15076	1.28911		19	10	0.88234	0.29765		80	2.5	0.1437	0.97674
28	5	5	0.1269	0.5569		0.625	20	0.61264	1.49846		37.5	10	0.83072	0.23576		5	5	0.64567	0.85208
29	5	10	0.12167	0.53886		1.25	20	0.39696	0.63126		75	10	0.81788	0.37189		10	5	0.58803	0.85449
30	5	20	0.16616	0.69199		2.5	20	0.18888	0.42216		150	10	0.51945	0.45866		20	5	0.50447	1.07892
31	10	0.3125	0.17985	1.47698		5	20	0.1365	0.60864		19	20	0.81079	0.19287		40	5	0.163	0.54987
32	10	0.625	0.15922	1.33554		10	20	0.14011	1.22439		37.5	20	0.76871	0.20866		80	5	0.11805	0.81594
33	10	1.25	0.16359	1.3655		0.625	40	0.41207	0.7388		75	20	0.71979	0.30893		5	10	0.69839	2.6076
34	10	2.5	0.1385	1.19355		1.25	40	0.28346	0.46922		150	20	0.42025	0.42155		10	10	0.59339	1.08133
35	10	5	0.14639	1.24771		2.5	40	0.17605	0.42683		19	40	0.69643	0.1223		20	10	0.51475	1.18474
36	10	10	0.148	1.25885		5	40	0.1461	0.66598		37.5	40	0.6102	0.14585		40	10	0.18007	0.60532
37	10	20	0.13289	1.15516		10	40	0.12329	1.11918		75	40	0.5581	0.24944		80	10	0.13476	0.92055
38											150	40	0.35127	0.39673		5	20	0.59294	1.13349
39																10	20	0.67784	3.9055
40																20	20	0.60813	2.42957
41																40	20	0.22248	0.74881
42																80	20	0.14929	1.01241
43																5	40	0.55585	1.21835
44																10	40	0.51206	1.00999
45																20	40	0.51296	1.54412
46																40	40	0.20979	0.70678
47																80	40	0.16443	1.10979
48																			

CT	CU	CV	CW	CX	CY	CZ	DA	DB	DC	DD	DE	DF	DG	DH
22Rv1					22Rv1					22Rv1				
Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC206 (nM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC212 (nM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index	
0.16	0.16	0.99999	123245		37.5	0.16	0.99999	1545493		2.5	0.16	0.99999	5668385	
0.3125	0.16	0.99851	15.1483		37.5	0.3125	0.99999	3018540		5	0.16	0.95602	0.52771	
0.625	0.16	1	7862927		37.5	0.625	0.91318	0.45253		10	0.16	0.99999	5668386	
1.25	0.16	0.74548	0.56018		37.5	1.25	0.87013	0.4034		20	0.16	0.99999	5668388	
2.5	0.16	0.21002	0.67502		37.5	2.5	0.87364	0.67215		40	0.16	0.99999	5668392	
5	0.16	0.1369	1.21091		37.5	5	0.85201	0.88337		80	0.16	0.20216	0.5086	
0.16	0.3125	0.99999	240713		37.5	10	0.92884	6.73873		2.5	0.3125	0.99999	1.11E+07	
0.3125	0.3125	0.99999	240714		37.5	20	0.82853	2.24968		5	0.3125	0.99999	1.11E+07	
0.625	0.3125	0.99999	240715		37.5	40	0.68383	1.0852		10	0.3125	0.99999	1.11E+07	
1.25	0.3125	0.72888	0.55072		75	0.16	0.99999	1545494		20	0.3125	0.99999	1.11E+07	
2.5	0.3125	0.1927	0.65988		75	0.3125	0.98527	4.67026		40	0.3125	0.89514	0.77479	
5	0.3125	0.12992	1.19561		75	0.625	0.96313	1.88993		80	0.3125	0.19314	0.50112	
0.16	0.625	0.99298	3.62622		75	1.25	0.92688	1.14291		2.5	0.625	0.98164	9.62963	
0.3125	0.625	0.98425	1.06019		75	2.5	0.98187	23.1255		5	0.625	0.9768	6.13557	
0.625	0.625	0.99999	481427		75	5	0.90731	2.27768		10	0.625	0.98824	23.3533	
1.25	0.625	0.69142	0.53065		75	10	0.92719	6.62972		20	0.625	0.99999	2.21E+07	
2.5	0.625	0.18923	0.65678		75	20	0.84706	3.01239		40	0.625	0.88144	0.82332	
5	0.625	0.13194	1.2001		75	40	0.72389	1.63434		80	0.625	0.1846	0.49394	
0.16	1.25	0.94573	0.26261		150	0.16	0.89403	0.72997		2.5	1.25	0.99999	4.43E+07	
0.3125	1.25	0.95127	0.40284		150	0.3125	0.75469	0.58734		5	1.25	0.9441	2.13011	
0.625	1.25	0.99	4.22687		150	0.625	0.75839	0.60466		10	1.25	0.98695	37.865	
1.25	1.25	0.6644	0.51821		150	1.25	0.76169	0.638		20	1.25	0.9905	70.7169	
2.5	1.25	0.19926	0.66574		150	2.5	0.76025	0.69649		40	1.25	0.82039	0.70294	
5	1.25	0.12707	1.18924		150	5	0.69176	0.66969		80	1.25	0.18492	0.49446	
0.16	2.5	0.99999	1925700		150	10	0.73018	0.91977		2.5	2.5	0.97535	21.3781	
0.3125	2.5	0.99999	1925701		150	20	0.63852	0.8474		5	2.5	0.94298	3.99988	
0.625	2.5	0.99999	1925702		150	40	0.53883	0.78929		10	2.5	0.99999	8.86E+07	
1.25	2.5	0.68227	0.53121		300	0.16	0.19444	0.73612		20	2.5	0.99275	239.167	
2.5	2.5	0.1818	0.65011		300	0.3125	0.17775	0.72217		40	2.5	0.85921	1.14772	
5	2.5	0.11671	1.16524		300	0.625	0.16612	0.71205		80	2.5	0.17848	0.48926	
0.16	5	0.9368	0.57369		300	1.25	0.17786	0.72258		2.5	5	0.94217	7.63406	
0.3125	5	0.95701	1.19993		300	2.5	0.16519	0.71176		5	5	0.94459	8.38049	
0.625	5	0.99999	3851402		300	5	0.15633	0.70423		10	5	0.97165	32.4604	
1.25	5	0.66014	0.5251		300	10	0.16313	0.71198		20	5	0.99999	1.77E+08	
2.5	5	0.16461	0.63394		300	20	0.15201	0.70371		40	5	0.76627	0.83325	
5	5	0.10941	1.14755		300	40	0.13471	0.68936		80	5	0.16479	0.47763	
0.16	10	0.89615	0.44622		600	0.16	0.11555	1.32298		2.5	10	0.90287	5.1341	
0.3125	10	0.9087	0.64199		600	0.3125	0.11833	1.32921		5	10	0.88064	3.32666	
0.625	10	0.93977	1.43576		600	0.625	0.11689	1.32604		10	10	0.9172	7.33611	
1.25	10	0.608	0.50538		600	1.25	0.10886	1.30772		20	10	0.90883	6.16828	
2.5	10	0.15763	0.62725		600	2.5	0.1033	1.29455		40	10	0.74871	1.04249	
5	10	0.10587	1.13877		600	5	0.10024	1.28727		80	10	0.15461	0.46927	
0.16	20	0.79932	0.25296		600	10	0.10391	1.29686		2.5	20	0.82571	2.78002	
0.3125	20	0.83911	0.44515		600	20	0.09399	1.27265		5	20	0.75789	1.28787	
0.625	20	0.87742	0.84619		600	40	0.08852	1.25949		10	20	0.83666	3.33606	
1.25	20	0.51096	0.46601							20	20	0.85486	4.4842	
2.5	20	0.13658	0.60563							40	20	0.60631	0.7169	
5	20	0.09144	1.10054							80	20	0.13764	0.4542	
0.16	40	0.56991	0.10904							2.5	40	0.56717	0.47527	
0.3125	40	0.58842	0.17581							5	40	0.51208	0.33888	
0.625	40	0.64716	0.34055							10	40	0.5886	0.63493	
1.25	40	0.2824	0.37198							20	40	0.57345	0.67086	
2.5	40	0.12281	0.59059							40	40	0.42332	0.48218	
5	40	0.08925	1.09459							80	40	0.11467	0.43142	

DI	DJ	DK	DL	DM	DN	DO	DP	DQ	DR
Hep3B					Hep3B				
Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index	
0.625	0.625	0.95079	0.69959		0.625	0.625	0.8784	0.83792	
0.625	1.25	0.99999	2.27532		0.625	1.25	0.82139	0.5959	
0.625	2.5	0.99999	2.2754		0.625	2.5	0.81307	0.57158	
0.625	5	0.99999	2.27557		0.625	5	0.90926	1.09467	
0.625	10	0.97992	2.75889		0.625	10	0.78524	0.50197	
0.625	20	0.99999	2.27655		0.625	20	0.85209	0.71623	
0.625	40	0.82226	120.654		0.625	40	0.66863	0.3205	
1.25	0.625	0.88686	1.54306		1.25	0.625	0.80853	1.11722	
1.25	1.25	0.92418	1.85647		1.25	1.25	0.75378	0.87675	
1.25	2.5	0.98661	1.19603		1.25	2.5	0.78857	1.01756	
1.25	5	0.8395	13.6255		1.25	5	0.86222	1.5062	
1.25	10	0.90745	13.9277		1.25	10	0.73109	0.80195	
1.25	20	0.84653	50.2817		1.25	20	0.79023	1.02694	
1.25	40	0.69423	259.289		1.25	40	0.5667	0.46192	
2.5	0.625	0.48067	11.1895		2.5	0.625	0.41576	0.58394	
2.5	1.25	0.4877	21.1786		2.5	1.25	0.38143	0.52417	
2.5	2.5	0.50008	39.6511		2.5	2.5	0.41258	0.57821	
2.5	5	0.46812	90.2371		2.5	5	0.44661	0.64202	
2.5	10	0.62042	92.8266		2.5	10	0.37084	0.50665	
2.5	20	0.52335	283.367		2.5	20	0.35345	0.4787	
2.5	40	0.41423	908.23		2.5	40	0.2984	0.39619	
5	0.625	0.34611	20.3652		5	0.625	0.30188	0.80234	
5	1.25	0.35967	37.3498		5	1.25	0.27783	0.7347	
5	2.5	0.37322	69.277		5	2.5	0.30989	0.82549	
5	5	0.36017	146.018		5	5	0.32713	0.87642	
5	10	0.41473	227.491		5	10	0.30339	0.80668	
5	20	0.39782	489.339		5	20	0.25771	0.68002	
5	40	0.28787	1657.13		5	40	0.19102	0.50857	
10	0.625	0.26862	30.5198		10	0.625	0.26195	1.38279	
10	1.25	0.31565	46.8449		10	1.25	0.26286	1.38769	
10	2.5	0.3287	86.1907		10	2.5	0.2677	1.4139	
10	5	0.32318	175.024		10	5	0.30021	1.59515	
10	10	0.34159	318.588		10	10	0.2993	1.58996	
10	20	0.3113	736.292		10	20	0.19495	1.03667	
20	40	0.18996	2977.7		10	40	0.13645	0.75148	

DS	DT	DU	DV	DW	DX	DY	DZ	EA	EB	EC	ED	EE	EF
SNB19					SNB19					SNB19			
Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC206 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index
0.625	20	0.82857	0.94632		0.625	0.16	0.99999	1.87E+32		0.3125	0.16	0.72662	3.87117
1.25	20	0.93279	3.42228		0.625	0.3125	0.99999	3.66E+32		0.3125	0.3125	0.70569	3.2735
2.5	20	0.65835	1.35886		0.625	0.625	0.99999	7.32E+32		0.3125	0.625	0.68999	2.89977
5	20	0.30751	0.83613		0.625	1.25	0.99999	1.46E+33		0.3125	1.25	0.71354	3.48296
10	20	0.22141	1.01348		0.625	2.5	0.99999	2.93E+33		0.3125	2.5	0.68345	2.75973
20	20	0.22192	1.76227		0.625	5	0.99999	5.86E+33		0.3125	5	0.70634	3.29038
40	20	0.2036	2.97407		0.625	10	0.99999	1.17E+34		0.3125	10	0.71812	3.61319
0.625	40	0.89558	1.74228		0.625	20	0.97972	0.21713		0.3125	20	0.77763	6.05441
1.25	40	0.90107	2.83806		0.625	40	0.77972	0.13044		0.3125	40	0.65082	2.17215
2.5	40	0.58718	1.41972		1.25	0.16	0.98462	0.45874		0.625	0.16	0.34866	0.56977
5	40	0.32614	1.16671		1.25	0.3125	0.96294	0.3848		0.625	0.3125	0.32583	0.48246
10	40	0.23583	1.34327		1.25	0.625	0.95455	0.36915		0.625	0.625	0.3259	0.48269
20	40	0.21962	2.01259		1.25	1.25	0.97483	0.41593		0.625	1.25	0.32989	0.49713
40	40	0.2059	3.27509		1.25	2.5	0.95664	0.37273		0.625	2.5	0.33604	0.52005
0.625	60	0.63735	1.20778		1.25	5	0.96643	0.39258		0.625	5	0.33434	0.51363
1.25	60	0.66116	1.5025		1.25	10	0.99999	1.17E+34		0.625	10	0.35481	0.59534
2.5	60	0.44664	1.37007		1.25	20	0.98462	0.45874		0.625	20	0.35428	0.59313
5	60	0.277	1.31721		1.25	40	0.76923	0.25784		0.625	40	0.29804	0.39069
10	60	0.21573	1.52633		2.5	0.16	0.69182	0.47743		1.25	0.16	0.28967	0.73171
20	60	0.19294	2.06726		2.5	0.3125	0.68336	0.47378		1.25	0.3125	0.25108	0.53191
40	60	0.16996	2.99993		2.5	0.625	0.6742	0.46991		1.25	0.625	0.24447	0.50208
0.625	80	0.19313	1.09608		2.5	1.25	0.68888	0.47615		1.25	1.25	0.24768	0.5164
1.25	80	0.28983	1.24369		2.5	2.5	0.68818	0.47585		1.25	2.5	0.2482	0.51877
2.5	80	0.25779	1.31961		2.5	5	0.6679	0.46731		1.25	5	0.25206	0.53644
5	80	0.1969	1.38485		2.5	10	0.71119	0.48612		1.25	10	0.26514	0.59948
10	80	0.15726	1.54059		2.5	20	0.71119	0.48612		1.25	20	0.27881	0.67081
20	80	0.13524	1.88364		2.5	40	0.61951	0.44845		1.25	40	0.25886	0.5686
40	80	0.10403	2.31734		5	0.16	0.4451	0.78127		2.5	0.16	0.25468	1.09731
0.625	100	0.00407	0.87397		5	0.3125	0.49196	0.81048		2.5	0.3125	0.24513	1.00996
1.25	100	0.00417	0.8773		5	0.625	0.44476	0.78105		2.5	0.625	0.23859	0.95297
2.5	100	0.00635	0.91978		5	1.25	0.44594	0.78178		2.5	1.25	0.24284	0.98976
5	100	0.00679	0.93309		5	2.5	0.44748	0.78273		2.5	2.5	0.24173	0.98005
10	100	0.00416	0.89199		5	5	0.44364	0.78036		2.5	5	0.22368	0.83124
20	100	0.00343	0.88654		5	10	0.4549	0.78731		2.5	10	0.23767	0.94518
40	100	0.00263	0.87999		5	20	0.44762	0.78282		2.5	20	0.21393	0.75754
					5	40	0.41028	0.75988		2.5	40	0.20628	0.70275
					10	0.16	0.38483	1.48859		5	0.16	0.24755	2.06325
					10	0.3125	0.39895	1.50589		5	0.3125	0.24748	2.06207
					10	0.625	0.38685	1.49107		5	0.625	0.24133	1.95327
					10	1.25	0.39853	1.50538		5	1.25	0.2501	2.10961
					10	2.5	0.40818	1.51719		5	2.5	0.26115	2.31891
					10	5	0.40406	1.51214		5	5	0.2571	2.24051
					10	10	0.39937	1.50641		5	10	0.24526	2.02225
					10	20	0.40755	1.51642		5	20	0.28757	2.87865
					10	40	0.35175	1.44787		5	40	0.21099	1.47231

EH	EI	EJ	EK	EL	EM	EN	EO	EP	EQ	ER	ES	ET	EU	EV	EW	EX	EY	EZ	FA
U251					U251					H1882					H1882				
Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index	
0.625	0.16	0.80769	0.27966		0.625	0.16	0.80769	0.27966		0.625	0.625	0.82745	0.60392		0.625	0.625	0.78794	67338.1	
1.25	0.16	0.96965	0.93284		1.25	0.16	0.96965	0.93284		0.625	1.25	0.81699	0.62206		0.625	1.25	0.88734	0.63353	
2.5	0.16	0.54308	0.79201		2.5	0.16	0.54308	0.79201		0.625	2.5	0.77516	0.44192		0.625	2.5	0.84891	151.766	
5	0.16	0.36699	1.37747		5	0.16	0.36699	1.37747		0.625	5	0.83007	1.89963		0.625	5	0.85553	117.196	
0.625	0.3125	0.73371	0.27251		0.625	0.3125	0.73371	0.27251		0.625	10	0.75098	0.45308		0.625	10	0.88072	4.76692	
1.25	0.3125	0.78168	0.53457		1.25	0.3125	0.78168	0.53457		0.625	20	0.74248	0.48986		0.625	20	0.82107	47223	
2.5	0.3125	0.51566	0.78463		2.5	0.3125	0.51566	0.78463		0.625	40	0.80915	4.69922		0.6235	40	0.93108	0.58569	
5	0.3125	0.35832	1.37298		5	0.3125	0.35832	1.37298		1.25	0.625	0.67647	0.59802		1.25	0.625	0.74089	7715245	
0.625	0.625	0.69091	0.29316		0.625	0.625	0.69091	0.29316		1.25	1.25	0.73464	0.69017		1.25	1.25	0.87939	1.57898	
1.25	0.625	0.74993	0.55941		1.25	0.625	0.74993	0.55941		1.25	2.5	0.70523	0.64213		1.25	2.5	0.71107	4.66E+08	
2.5	0.625	0.52867	0.81238		2.5	0.625	0.52867	0.81238		1.25	5	0.7902	1.02439		1.25	5	0.77469	2193942	
5	0.625	0.35524	1.37966		5	0.625	0.35524	1.37966		1.25	10	0.72418	0.70296		1.25	10	0.77932	2702754	
0.625	1.25	0.72909	0.40254		0.625	1.25	0.72909	0.40254		1.25	20	0.70588	0.67716		1.25	20	0.71438	2.78E+09	
1.25	1.25	0.73161	0.63227		1.25	1.25	0.73161	0.63227		1.25	40	0.72941	0.85087		1.25	40	0.79655	1670964	
2.5	1.25	0.53063	0.85316		2.5	1.25	0.53063	0.85316		2.5	0.625	0.28601	0.54169		2.5	0.625	0.36289	3.71E+19	
5	1.25	0.35371	1.39799		5	1.25	0.35371	1.39799		2.5	1.25	0.3634	0.64181		2.5	1.25	0.3943	6.62E+18	
0.625	2.5	0.67007	0.48641		0.625	2.5	0.67007	0.48641		2.5	2.5	0.32582	0.59262		2.5	2.5	0.36057	1.78E+20	
1.25	2.5	0.6828	0.72129		1.25	2.5	0.6828	0.72129		2.5	5	0.34137	0.61282		2.5	5	0.36488	2.54E+20	
2.5	2.5	0.4807	0.87771		2.5	2.5	0.4807	0.87771		2.5	10	0.34712	0.62034		2.5	10	0.39848	3.86E+19	
5	2.5	0.32825	1.40256		5	2.5	0.32825	1.40256		2.5	20	0.31248	0.57546		2.5	20	0.36117	1.36E+21	
0.625	5	0.6649	0.74502		0.625	5	0.6649	0.74502		2.5	40	0.29059	0.5475		2.5	40	0.34374	1.08E+22	
1.25	5	0.7014	1.06257		1.25	5	0.7014	1.06257		5	0.625	0.13601	0.69268		5	0.625	0.13108	1.03E+30	
2.5	5	0.49538	1.03128		2.5	5	0.49538	1.03128		5	1.25	0.14784	0.72568		5	1.25	0.14751	1.72E+29	
5	5	0.33175	1.48095		5	5	0.33175	1.48095		5	2.5	0.13444	0.68824		5	2.5	0.13068	4.39E+30	
0.625	10	0.6579	1.24417		0.625	10	0.6579	1.24417		5	5	0.14418	0.71555		5	5	0.13605	3.79E+30	
1.25	10	0.69664	1.65548		1.25	10	0.69664	1.65548		5	10	0.14739	0.72442		5	10	0.14692	1.50E+30	
2.5	10	0.4793	1.26696		2.5	10	0.4793	1.26696		5	20	0.14444	0.71628		5	20	0.1281	5.32E+31	
5	10	0.34098	1.64947		5	10	0.34098	1.64947		5	40	0.13876	0.70041		5	40	0.12087	3.53E+32	
0.625	20	0.6607	2.29956		0.625	20	0.6607	2.29956		10	0.625	0.12157	1.30246		10	0.625	0.1837	7.40E+26	
1.25	20	0.69902	2.89708		1.25	20	0.69902	2.89708		10	1.25	0.14209	1.41946		10	1.25	0.16832	1.01E+28	
2.5	20	0.48378	1.80777		2.5	20	0.48378	1.80777		10	2.5	0.13712	1.39163		10	2.5	0.16024	5.86E+28	
5	20	0.31343	1.85783		5	20	0.31343	1.85783		10	5	0.15673	1.50003		10	5	0.17369	2.03E+28	
0.625	40	0.72685	5.79782		0.625	40	0.72685	5.79782		10	10	0.14556	1.43871		10	10	0.16329	1.56E+29	
1.25	40	0.71818	5.80153		1.25	40	0.71818	5.80153		10	20	0.13562	1.38315		10	20	0.15229	1.40E+30	
2.5	40	0.47021	2.75561		2.5	40	0.47021	2.75561		10	40	0.10954	1.23104		10	40	0.12081	3.57E+32	
5	40	0.37818	2.80331		5	40	0.37818	2.80331											

FB	FC	FD	FE	FF	FG	FH	FI	FJ	FK	FL	FM	FN	FO
H1048					H1048					H1048			
Dose of ONC201 (μM)	Dose of Tazemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC201 (μM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index		Dose of ONC206 (nM)	Dose of valemetostat (μM)	Relative cell viability	Combination Index
0.625	0.625	0.99999	149.911		0.3125	0.16	0.99999	533503		37.5	0.16	0.99999	2163.45
0.625	1.25	0.99043	2.80279		0.625	0.16	0.90176	0.50461		75	0.16	0.99999	2164.18
0.625	2.5	0.99999	150.856		1.25	0.16	0.48172	0.57659		150	0.16	0.99999	2165.66
0.625	5	0.99999	152.116		2.5	0.16	0.24968	0.90444		300	0.16	0.37974	0.6473
0.625	10	0.99999	154.636		5	0.16	0.20364	1.69971		600	0.16	0.19636	1.09304
0.625	20	0.99999	159.675		0.3125	0.3125	0.99999	1041996		37.5	0.3125	0.99999	4224.77
0.625	40	0.94183	0.96609		0.625	0.3125	0.9293	0.57548		75	0.3125	0.99999	4225.51
1.25	0.625	0.93152	1.73182		1.25	0.3125	0.45848	0.5641		150	0.3125	0.97702	0.70946
1.25	1.25	0.90869	1.4454		2.5	0.3125	0.2372	0.89009		300	0.3125	0.37601	0.64541
1.25	2.5	0.95508	2.24323		5	0.3125	0.19619	1.68108		600	0.3125	0.20009	1.09778
1.25	5	0.91311	1.49221		0.3125	0.625	0.93547	0.36361		37.5	0.625	0.99999	8448.81
1.25	10	0.93962	1.87395		0.625	0.625	0.83981	0.44628		75	0.625	0.99999	8449.55
1.25	20	0.93004	1.71248		1.25	0.625	0.43066	0.54939		150	0.625	0.94226	0.59345
1.25	40	0.6768	0.58779		2.5	0.625	0.22171	0.87186		300	0.625	0.35601	0.63524
2.5	0.625	0.44912	0.67946		5	0.625	0.19576	1.68002		600	0.625	0.20531	1.10434
2.5	1.25	0.43697	0.66038		0.3125	1.25	0.99426	21.1366		37.5	1.25	0.99999	16896.9
2.5	2.5	0.45751	0.69294		0.625	1.25	0.85501	0.47725		75	1.25	0.97672	0.3624
2.5	5	0.41208	0.62261		1.25	1.25	0.44672	0.5583		150	1.25	0.92704	0.56697
2.5	10	0.41649	0.62927		2.5	1.25	0.23749	0.8905		300	1.25	0.39152	0.65326
2.5	20	0.40619	0.61408		5	1.25	0.19504	1.67824		600	1.25	0.20098	1.09891
2.5	40	0.3028	0.47227		0.3125	2.5	0.94636	0.80097		37.5	2.5	0.99999	33793
5	0.625	0.3268	1.00668		0.625	2.5	0.80611	0.44256		75	2.5	0.96031	0.32568
5	1.25	0.31141	0.96625		1.25	2.5	0.40227	0.53509		150	2.5	0.92137	0.55937
5	2.5	0.25803	0.82997		2.5	2.5	0.22444	0.87525		300	2.5	0.34557	0.6299
5	5	0.30515	0.95001		5	2.5	0.18658	1.65669		600	2.5	0.20486	1.10378
5	10	0.33343	1.02438		0.3125	5	0.9511	1.53031		37.5	5	0.99999	67585.3
5	20	0.29293	0.9186		0.625	5	0.81557	0.48846		75	5	0.99999	67586.1
5	40	0.22614	0.7507		1.25	5	0.40198	0.53589		150	5	0.96165	0.65655
10	0.625	0.22585	1.49946		2.5	5	0.223	0.87373		300	5	0.34766	0.63098
10	1.25	0.24462	1.59287		5	5	0.19532	1.67917		600	5	0.20203	1.10023
10	2.5	0.2472	1.60574		0.3125	10	0.97677	11.2152		37.5	10	0.99999	135170
10	5	0.23277	1.53387		0.625	10	0.85473	0.69408		75	10	0.99999	135171
10	10	0.26119	1.67588		1.25	10	0.41532	0.54526		150	10	0.97702	0.7872
10	20	0.23358	1.53799		2.5	10	0.22903	0.88129		300	10	0.37795	0.64642
10	40	0.16856	1.21484		5	10	0.19317	1.67398		600	10	0.20352	1.10211
					0.3125	20	0.94206	3.78415		37.5	20	0.99999	270339
					0.625	20	0.82533	0.74909		75	20	0.98657	0.83159
					1.25	20	0.39825	0.53948		150	20	0.98001	0.93439
					2.5	20	0.20077	0.84744		300	20	0.36467	0.63971
					5	20	0.17998	1.6403		600	20	0.18412	1.07717
					0.3125	40	0.85386	1.20241		37.5	40	0.96344	0.29453
					0.625	40	0.73426	0.60531		75	40	0.91793	0.30364
					1.25	40	0.33486	0.50753		150	40	0.87839	0.52213
					2.5	40	0.16521	0.80136		300	40	0.32334	0.61849
					5	40	0.14513	1.54394		600	40	0.14658	1.02433

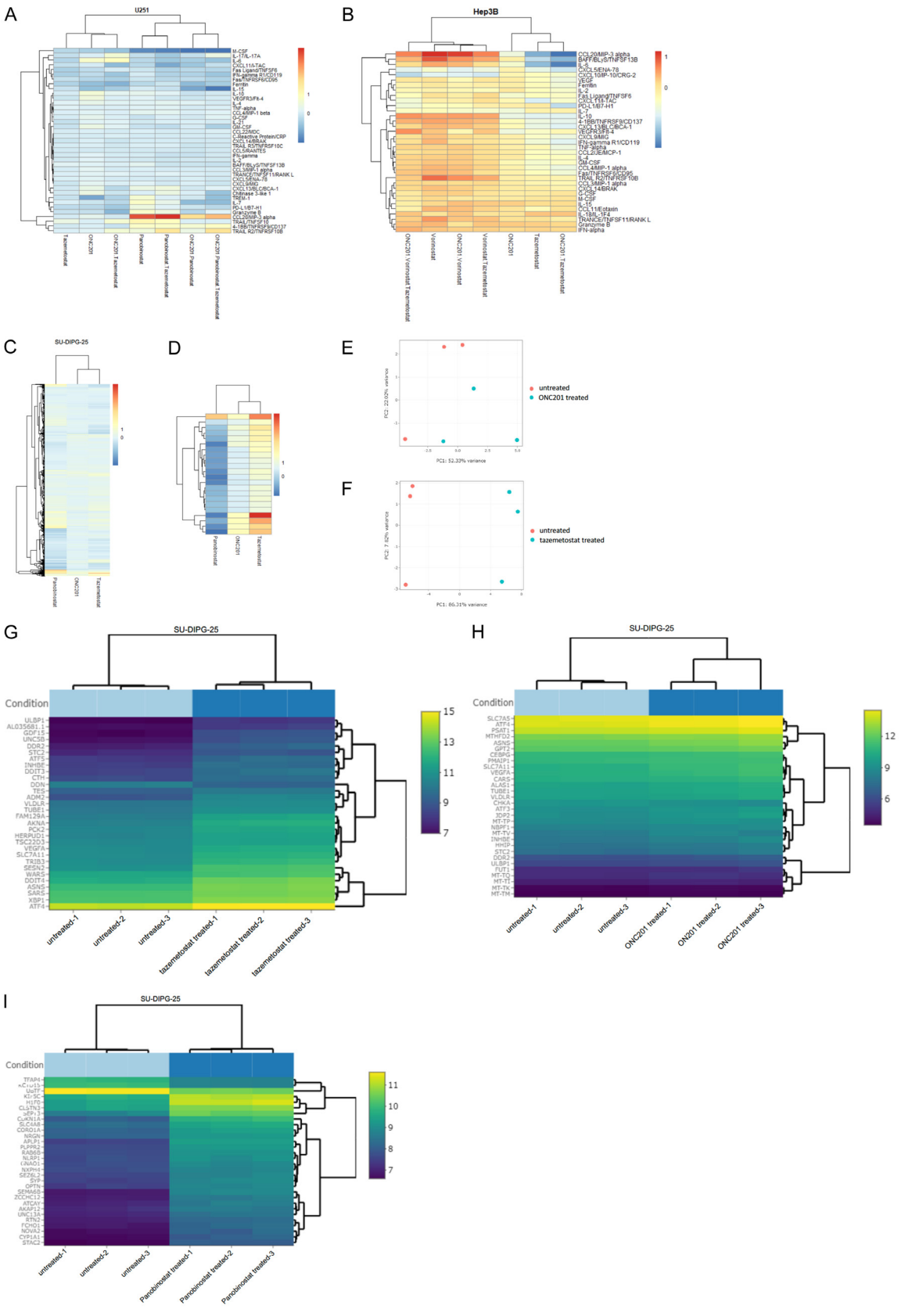

A
U251
B
Hep3B
C
SU-DIPG-25
D
E
untreated
ONC201 treated
F
untreated
tazemetostat treated
G
SU-DIPG-25
Condition
untreated-1
untreated-2
untreated-3
tazemetostat treated-1
tazemetostat treated-2
tazemetostat treated-3
H
SU-DIPG-25
Condition
untreated-1
untreated-2
untreated-3
ONC201 treated-1
ONC201 treated-2
ONC201 treated-3
I
SU-DIPG-25
Condition
untreated-1
untreated-2
untreated-3
Panobinostat treated-1
Panobinostat treated-2
Panobinostat treated-3

Figure S4. ONC201 has similar e:ects as EZH2 inhibitor on cytokine and transcriptional profiles in tumor cells. (A, B) Cytokine profile assay of (A) U251 GBM cells upon treatment with ONC201, tazemetostat or Panobinostat alone or the indicated combinations, and (B) Hep3B HCC cells upon the treatment with ONC206, tazemetostat or Panobinostat alone or the indicated combinations. (C) Cluster heatmap of mRNA expression profiles of SU-DIPG-25 cells treated with ONC201, tazemetostat or Panobinostat alone by plotting their log2FoldChange transformed expression values. (D) Cluster heatmap of mRNA expression profiles of diUerentially expressed genes with adjusted *p*-value < 0.05 in SU-DIPG-25 cells treated with ONC201, tazemetostat or Panobinostat alone by plotting their log2FoldChange transformed expression values. (E, F) The principal component analysis revealing the similarities between untreated SU-DIPG-25 cells and SU-DIPG-25 cells treated with ONC201 (E) or tazemetostat (F). (G-I) Bi-clustering heatmaps indicating the expression profile of the top 30 differentially expressed genes sorted by their adjusted *p*-value by plotting their log2 transformed expression values in SU-DIPG-25 cells treated by drugs indicated in the plot. Each group was conducted with triplicates.

Table S3. The top 30 differentially expressed genes in tazemetostat, ONC201 or Panobinostat treated SU-DIPG-25 cells

Top 30 differentially expressed genes in tazemetostat treated SU-DIPG-25 cells	Top 30 differentially expressed genes in ONC201 treated SU-DIPG-25 cells	Top 30 differentially genes in panobinostat treated SU-DIPG-25 cells
ADM2	ALAS1	AKAP12
AKNA	ASNS	APLP1
AL035681.1	ATF3	ATCAY
ASNS	ATF4	CDKN1A
ATF4	CARS	CLSTN3
ATF5	CEBPG	CORO1A
CTH	CHKA	CYP1A1
DDIT3	DDR2	FCHO1
DDIT4	FUT1	GNAO1
DDN	GPT2	H1F0
DDR2	HHIP	KCTD15
FAM129A	INHBE	KIF5C
GDF15	JDP2	NLRP1
HERPUD1	MTHFD2	NOVA2
INHBE	MT-TI	NRGN
PCK2	MT-TK	NXPH4
SARS	MT-TM	OPTN
SESN2	MT-TP	PLPPR2
SLC7A11	MT-TQ	RAB6B
STC2	MT-TV	RTN2
TES	NBPF1	SEMA6B
TRIB3	PMAIP1	SEPT3
TSC22D3	PSAT1	SEZ6L2
TUBE1	SLC7A11	SLC4A8
ULBP1	SLC7A5	STAC2
UNC5B	STC2	SYP
VEGFA	TUBE1	TFAP4
VLDLR	ULBP1	UBTF
WARS	VEGFA	UNC13A
XBP1	VLDLR	ZCCHC12