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Original Article

Dopamine pre-treatment impairs the anti-cancer effect of integrated stress response- and TRAIL pathway-inducing ONC201, ONC206 and ONC212 imipridones in pancreatic, colorectal cancer but not DMG cells

Yiqun Zhang[1,2,3,4], Nikos Tapinos[2,3,5], Rishi Lulla[1,2,6], Wafik S El-Deiry[1,2,3,4,7]

[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]Division of Hematology/Oncology, Department of Medicine, Lifespan and Brown University, Providence, RI, USA

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Abstract: ONC201 (originally discovered as TRAIL-Inducing Compound #10 or TIC10) and analogue ONC206 have been found to induce an integrated stress response with suggested primary targets and mechanisms involving targeting mitochondrial protein ClpP and antagonism of dopamine receptors D2/3 (DRD2/3). We hypothesized that dopamine, the agonist of DRD2, may counteract ONC201 or ONC206 for DRD2/3 and impair the anti-cancer effect of ONC201 or ONC206, thus protect the tumor cells from the cytotoxic effect of ONC201 or ONC206. We therefore pre-treated cancer cells from different tissue origins including breast cancer, pancreatic cancer, colorectal cancer, and diffuse midline glioma (DMG) with dopamine, followed by treatment of ONC201, ONC206 or ONC212. We observed that 48 hours of pre-treatment with dopamine impaired the cell viability suppression effect of ONC201, ONC206 and ONC212 in pancreatic cancer cells and colorectal cancer cells. We pre-treated multiple cancer cell lines with dopamine for one week followed by ONC201, ONC206, or ONC212 treatment and performed colony assays. Pre-treatment with dopamine impaired the anti-cancer effect of ONC201 or ONC206 in pancreatic cancer and colorectal cancer. Impairment of ONC212 effect by pre-treatment with dopamine was also seen in colony assay for colorectal cancer, but not in pancreatic cancer cells by colony assay. No protection from killing by imipridones was observed with DRD2 agonist sumanirole in tumor cells, or with brain tumor cell lines pretreated with dopamine. Immunoblotting was conducted to investigate whether dopamine pre-treatment impacts signaling pathways reported to be affected by ONC201. The dopamine pre-treatment did not impact changes in ATF4, CHOP, DR5 and ClpX which were reported to be affected by ONC201. The mechanism of impairment of ONC201/206/212 effect caused by dopamine pre-treatment appears to involve upregulation of anti-apoptotic p-Bad, XIAP, FLIP and pAkt. Our results shed light on mechanisms of cancer cell protection by dopamine after imipridone treatment, heterogeneity among different tumor cell types, and suggest that effects of dopamine adaptation on tumor cells may impact on cell survival pathways in ways that may or may not depend on expression of dopamine receptors.

Keywords: ONC201, ONC206, ONC212, imipridone, dopamine, integrated stress response, pancreatic cancer, colorectal cancer, diffuse midline glioma

Introduction

ONC201, the founding member of the imipridone class of drugs, was originally discovered as TRAIL-Inducing compound #10 or TIC10. ONC201 and analogues ONC206 and ONC212 are promising anti-cancer agents based on clinical activity against H3K27M-mutated diffuse midline gliomas (DMG), and other dopamine receptor DRD2/DRD3 overexpressing tumors

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such as neuroendocrine tumors without H3K-27M mutation. ONC201 inactivates the cell proliferation- and cell survival-promoting Akt and ERK kinases resulting in Foxo3a transcription factor translocation to the nucleus and upregulation of the death ligand TRAIL as an important mechanism of the cytotoxic effect of imipridones [1].

The mechanism of the action of imipridones also involve activation of eIF2α-ATF4 pathway eliciting the cellular integrated stress response (ISR). The upregulation of ATF4 is required for the increased abundance of TRAIL receptor, DR5 induced by ONC201 [2], and thus imipridones increase TRAIL and its receptor DR5. In addition, imipridones bind with and activate mitochondrial caseinolytic protease ClpP resulting in impairment of oxidative phosphorylation to kill tumor cells through apoptosis [3, 4].

ONC201 and ONC206 are D2-like dopamine receptor (DRD2 and DRD3) antagonists [5, 6]. Dopamine receptors belong to the superfamily of G-protein-coupled-receptors (GPCRs). Agonism of D2-like dopamine receptors coupled with Gi protein leads to reduction of cyclic AMP and less protein kinase A (PKA) activity.

We hypothesized that the DRD2 agonist dopamine may counteract ONC201/ONC206 antagonism of the receptor and protect the tumor cells from the cytotoxic effect of ONC201/ONC206. We therefore pre-treated tumor cells with dopamine to induce adaptation to dopamine followed by treatment with ONC201/ONC206/ONC212. We observed that dopamine pre-treatment protected tumor cells partially from the cytotoxic effect of ONC201/ONC206/ONC212 in certain types of tumor cells.

Materials and methods

Cell culture and reagents

All DMG cell lines including SU-DIPG-4, SU-DIPG-13, and SU-DIPG-36 cell lines originated at Stanford university and were generously provided by Dr. M. Monje to our group. The cells were maintained in NeurobasalTM-A Medium, enriched with Antibiotic-Antimycotic liquid, B-27 supplement minus vitamin A, sodium pyruvate solution, non-essential amino acids solution, glutaMAX, HEPES Buffer solution (all purchased from Thermo Fisher Scientific Inc., Invitrogen brand, Carlsbad, CA, USA), 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). The human CRC, lung cancer, GBM, PDAC cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in their ATCC-recommended media supplemented with 10% (v/v) fetal bovine serum and 1% Penicillin/Streptomycin. All cancer cell lines were confirmed to be mycoplasma free using PCR testing methods and were cultured at 37°C within a 95% humidified atmosphere containing 5% carbon dioxide in an incubator. Dopamine hydrochloride was purchased from Millipore Sigma and was solubilized in PBS at a storage concentration of 50 mM. ONC201, ONC206 and ONC212 were supplied by Chimerix, Inc and reconstituted in DMSO at a storage concentration of 20 mM. Sumanirole was purchased from TOCRIS biothechne and reconstituted in DMSO at a storage concentration of 20 mM.

Cell viability assays

Cells were seeded in opaque-walled 96-well plates at a density of 5000 cells per well and incubated overnight in 100 µL culture medium before addition of dopamine or sumanirole. The cells were pre-treated with dopamine or sumanirole for 48 hours followed by treatment of dopamine or sumanirole plus ONC201/ONC201/ONC212 for another 72 hours. 20 µL CellTiter-Glo bioluminescence agent (Promega Corporation, Madison, WI) was added into each well. The content was mixed for 2 minutes on a plate shaker to induce cell lysis. Cell viability was determined with the CellTiter-Glo assay. Each experiment was conducted in triplicate except for sumanirole pretreatment experiment.

Colony assays

A total of 1000 BxPC3 or 2000 HT29 cells/well were plated in 6-well plates. After 48-hour incubation of BxPC3 cells and 24-hour incubation of HT29 cells, medium was replaced with that containing dopamine. The medium and dopamine or sumanirole was changed every 3-4 days. After 7 days of treatment with dopamine

or sumanirole alone, fresh medium with dopamine or sumanirole and ONC201/ONC206/ONC212 was added. Cells were cultured for 3-7 days, with replenishment of medium and agents every 1 to 3 days until the cells not pretreated with dopamine or sumanirole were killed by ONC201/ONC206/ONC212. Cells were washed once with PBS, fixed with 10% formalin for 15 minutes, and stained with crystal violet for 30 minutes.

Immunoblotting

Cells were seeded in 6-well plates at a density of 5×10^4 cells per well for BxPC3 and 1×10^5 per well for HT29 and incubated overnight in culture media before the addition of dopamine or sumanirole. The culture was continued for 7 days with replenishment of medium after 1 to 3 days depending on the acidity of the medium. After 7 days of pre-treatment with dopamine alone, fresh medium with dopamine and ONC201/ONC206/ONC212 was added. Cells were cultured for 1-3 days, with replenishment of medium daily. 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 (pH8.0)]. The proteins were quantified with the Bio-Rad protein assay. LDS Sample Buffer (4X) and reducing reagent were added into the lysates. 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 Cleaved PARP (Asp214) (19F4) (cat. no. 9546S, Cell Signaling), ATF4 (cat. no. 11815S, Cell Signaling), CHOP (cat. no. 2895S, Cell Signaling), DR5 (cat. no. 3696S, Cell Signaling), ClpX (SKU. HPA040262, Millipore Sigma), pBAD (cat. no. 4366S, Cell Signaling), BAD (cat. no. 9292S, Cell Signaling), BID (cat. no. 2002S, Cell Signaling), FLIP (cat. no. 56343S, Cell Signaling), XIAP (cat. no. 2042S, Cell Signaling), β-Actin (A5441, Millipore Sigma), Ran (cat. no. 610341, BD Bioscience). Secondary antibodies were acquired from Pierce (cat. nos. 31430 and 31460) (horseradish peroxide-conjugated).

Statistical analysis

GraphPad Prism 10.1.1 was used to analyze the data generated by CellTiter-Glo assay. Two-way ANOVA was used to compare the cell viability data generated in CellTiter-Glo assay. The difference was considered significant if $P<0.05$.

Results

Adaptation to dopamine partially protects tumor cells from cytotoxic effects of imipridones

To test our hypothesis that as a DRD2 agonist, dopamine may counteract the effects of ONC201/ONC206 on tumor cells and protect them from being killed by ONC201/ONC206, we treated tumor cells from different tissue origins with a combination of dopamine and ONC201/ONC201/ONC212. We reported no protection was observed in cancer cells including breast cancer, PDAC, lung cancer, CRC, GBM and DMG cells when we treated the cells with dopamine and ONC201 simultaneously for 72 hours [7].

We speculated that some tumor cells may exist in an environment containing dopamine *in vivo* and may have already been adapted to dopamine, which is different from *in vitro* conditions, we pre-treated tumor cells with dopamine alone for 48 hours followed by treatment of ONC201/ONC206/ONC212 in the presence of dopamine for 72 hours and assessed the cell viability with the Cell Titer Glo assay. We observed higher luminescent signals in pancreatic cancer cells BxPC3 and colorectal cancer cells HT29 treated with high dose range of dopamine and ONC201/206/212. Thus, pancreatic cancer cells BxPC3 and colorectal cancer cells HT29 may be protected partially from the cytotoxic effect of ONC201/206/212 at the high dose range of dopamine and ONC201/206/212 (**Figure 1**), though the protection was not significant by ANOVA comparison. No protection was observed in MCF7 and MDA-MB-468 breast cancer cells, T98G and U251 GBM cells (Supplementary Figure 1).

H3K27M-mutated diffuse midline glioma (DMG) cells which are sensitive to imipridones

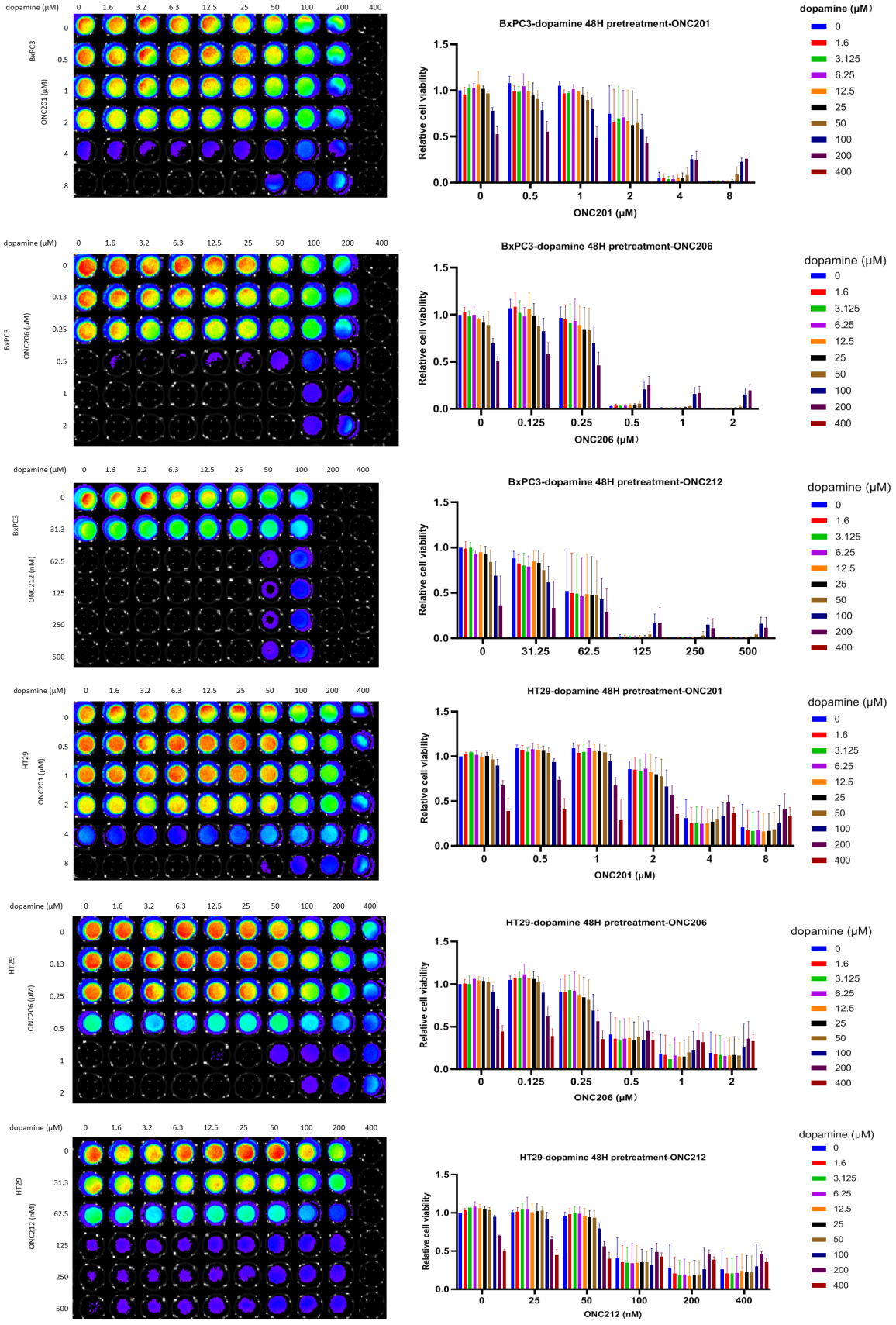
dopamine (μM) 0 1.6 3.2 6.3 12.5 25 50 100 200 400
BxPC3
ONC201 (μM) 0 0.5 1 2 4 8
BxPC3-dopamine 48H pretreatment-ONC201
Relative cell viability
ONC201 (μM)
dopamine (μM)
0 1.6 3.125 6.25 12.5 25 50 100 200 400
BxPC3
ONC206 (μM) 0 0.13 0.25 0.5 1 2
BxPC3-dopamine 48H pretreatment-ONC206
ONC206 (μM)
BxPC3
ONC212 (nM) 0 31.3 62.5 125 250 500
BxPC3-dopamine 48H pretreatment-ONC212
HT29
ONC201 (μM) 0 0.5 1 2 4 8
HT29-dopamine 48H pretreatment-ONC201
HT29
ONC206 (μM) 0 0.13 0.25 0.5 1 2
HT29-dopamine 48H pretreatment-ONC206
HT29
ONC212 (nM) 0 31.3 62.5 125 250 500
HT29-dopamine 48H pretreatment-ONC212
ONC212 (nM)

Figure 1. Impact of dopamine pre-treatment on the cytotoxic effect of imipridones by CellTiter-Glo assay. Cell Titer Glo assay of the viability of BxPC3 and HT29 cell lines pre-treated with dopamine for 48 H followed by dopamine and ONC201/ONC206/ONC212 for 72 H is shown. Drug doses and cell lines are as indicated.

normally grow in the brain in an environment with high levels of dopamine *in vivo*. Thus, we speculated that H3K27M-mutated DMG cells may be adapted to dopamine and antagonism to dopamine receptor may be the mechanism of their sensitivity to imipridones. Given these presumptions, we pre-treated H3K27M-mutated SU-DIPG-4 and SU-DIPG-13 human DMG cell lines with dopamine alone to simulate *in vitro* DMG cellular adaptation to dopamine followed by treatment with ONC201 in the presence of dopamine. However, no protection from the cytotoxic effect of ONC201 was observed by CTG analysis (Supplementary Figure 1).

We performed colony assays with prolonged dopamine pre-treatment to allow tumor cells to adapt to dopamine. We pretreated tumor cells with dopamine for 7 days, and then added ONC201/206/212 with or without the presence of dopamine and treated the cells for another 3-7 days. The duration of the ONC201/206/212 treatment depended on the tumor cells' response to the imipridones. After we treated BxPC3 cells with ONC201 or ONC206, we observed more colonies of cells pre-treated with 2 μM or 10 μM dopamine than when cells were not pre-treated with dopamine (**Figure 2A-D**), and this was not observed when we treated the cells with ONC212 (**Figure 2E**). Thus, cellular adaptation to dopamine protected BxPC3 cells from the cytotoxic effect of ONC201/206 but not ONC212 in colony assays. The protection by dopamine pre-treatment occurred even if we removed dopamine when the treatment with ONC201/ONC206 was started (**Figure 2B**, **2C**).

We also pre-treated HT29 cells with dopamine and more colonies of the cells pre-treated with dopamine were observed than when the tumor cells were not pre-treated with dopamine after we treated the cells with ONC201/206/212 (**Figure 2F-H**). Thus, dopamine pre-treatment protected HT29 cells from the cytotoxic effect of ONC201/206/212 as observed in colony assays. It was unexpected to observe that dopamine counteracted the cytotoxic effect of ONC212 as well which has never been reported as an antagonist of dopamine receptors DRD2 or DRD3.

No protection from the cytotoxic effect of ONC201 by pre-treatment with dopamine was observed in breast cancer MCF7 breast cancer cells, U251 GBM cells and SU-DIPG-4 and SU-DIPG-36 DMG cells by colony assays (**Figure 3**). Thus, protection from the cytotoxic effect of imipridones by dopamine presented heterogeneity among different types of cancers.

Pre-treatment with DRD2 agonist sumanirole could not protect tumor cells from the cytotoxic effect of imipridones

We investigated whether the protection provided by dopamine to some tumor cell lines treated with imipridones may be due to the agonism of DRD2, we pre-treated the tumor cells with sumanirole which is a selective DRD2 agonist. We treated cells for 48 hours with sumanirole followed by treatment with ONC201/206/ONC212 for 72 hours in the presence of continuous sumanirole treatment and then performed a CellTiter-Glo assay to assess cell viability. No protection against the imipridones was observed in BxPC3 or HT29 cells (**Figure 4**). We pre-treated the BxPC3 cells with sumanirole for 7 days followed by treatment of ONC201/206/212 for another 3 days in the presence of continuous sumanirole treatment, and sumanirole did not protect the tumor cells from the cytotoxic effect of ONC201/206/ONC212 in BxPC3 human pancreatic cancer cells (**Figure 5**).

Dopamine pre-treatment does not impact changes in ATF4, CHOP, DR5 and ClpX induced by ONC201

Imipridones kill tumor cells by triggering an integrated stress response dependent on ATF4 activation [8]. ATF4 promotes apoptosis, in part, by regulating the expression of CHOP [9]. CHOP induces TRAIL death receptor DR5 that plays a role in the induction of apoptosis under ER stress [10]. ATF4, CHOP and DR5 increase following treatment with ONC201. The mechanism of ONC201's effect also involves targeting the mitochondrial caseinolytic protease P (ClpP). With binding to ONC201, ClpP is activated without the requirement to bind with its chaperone, ClpX [3]. ClpP expression is

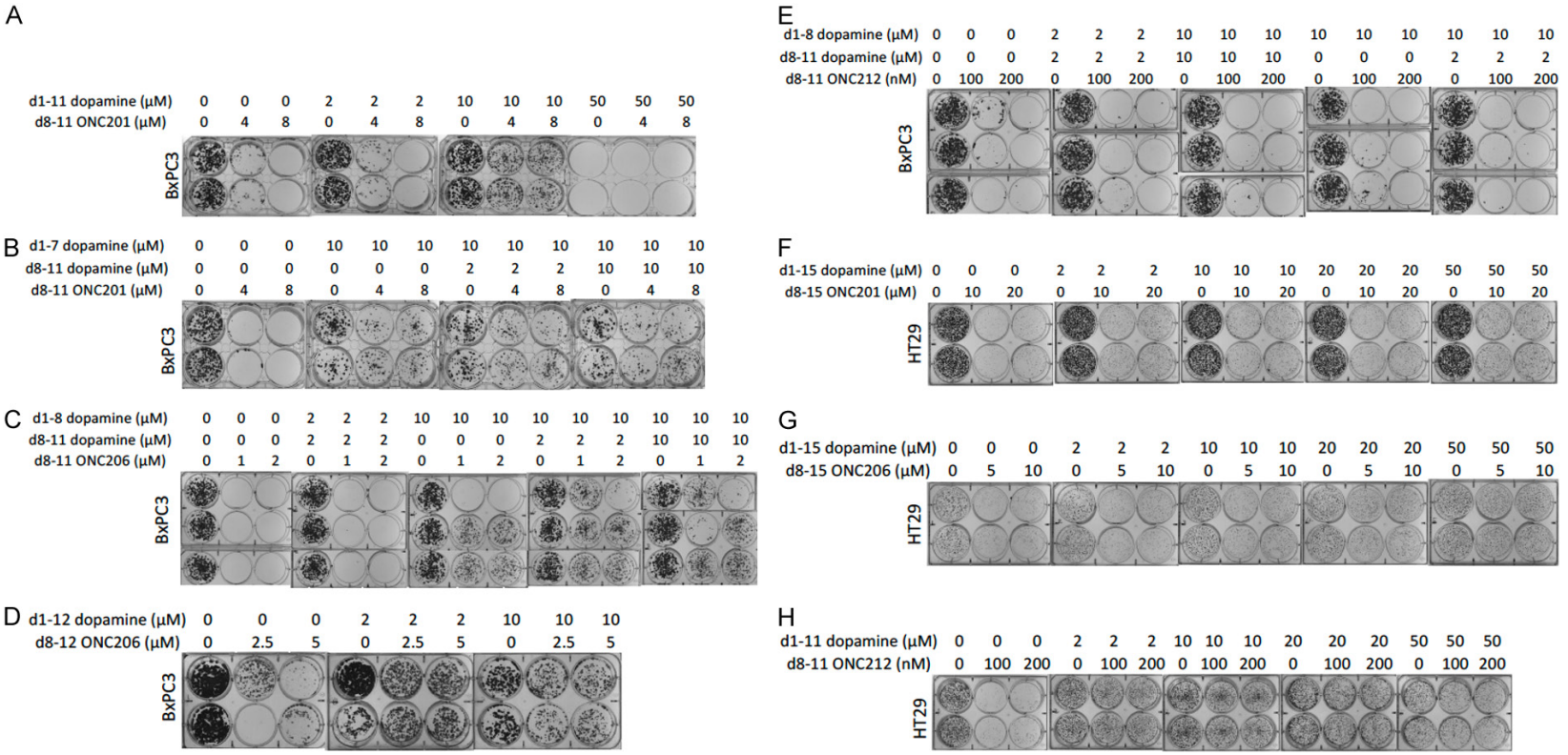

Figure 2. Impact of dopamine pre-treatment on the cytotoxic effect of imipridones in PDAC and CRC cells by colony assay. Colony assay of the viability of BxPC3 and HT29 cell lines pre-treated with dopamine for 7 days followed by dopamine and ONC201/ONC206/ONC212 treatment is shown. Drug doses, treatment duration and cell lines are as indicated.

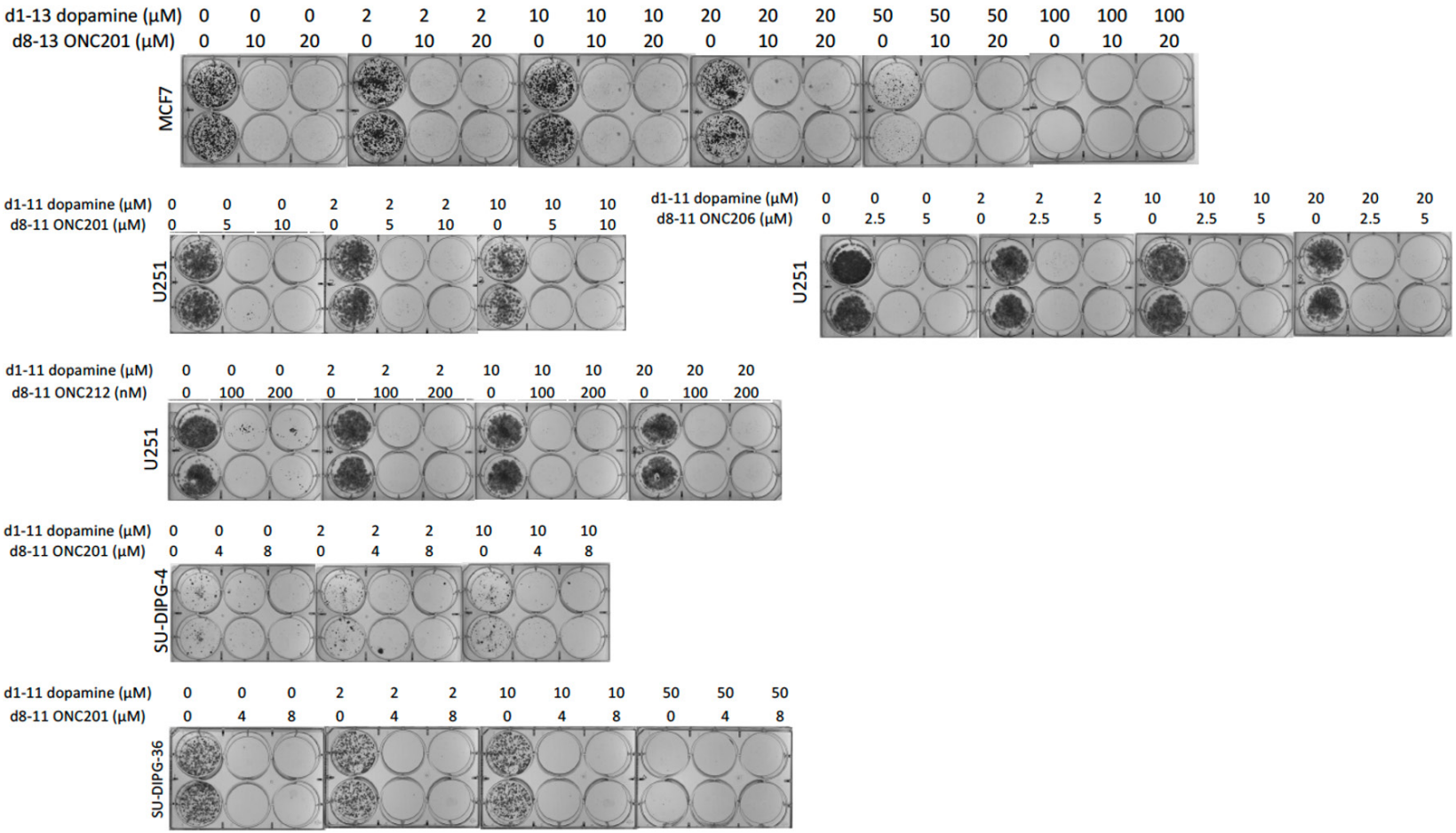

Figure 3. Impact of dopamine pre-treatment on the cytotoxic effect of imipridones in breast cancer, GBM and DMG cells by colony assay. Colony assay of the viability of MCF7, U251, SU-DIPG-4 and SU-SIPG-36 cells were pre-treated with dopamine for 7 days followed by dopamine and ONC201 for MCF7, SU-DIPG-4 and SU-SIPG-36 and dopamine and ONC201/ONC206/ONC212 for U251 is shown. Drug doses, treatment duration and cell lines are as indicated.

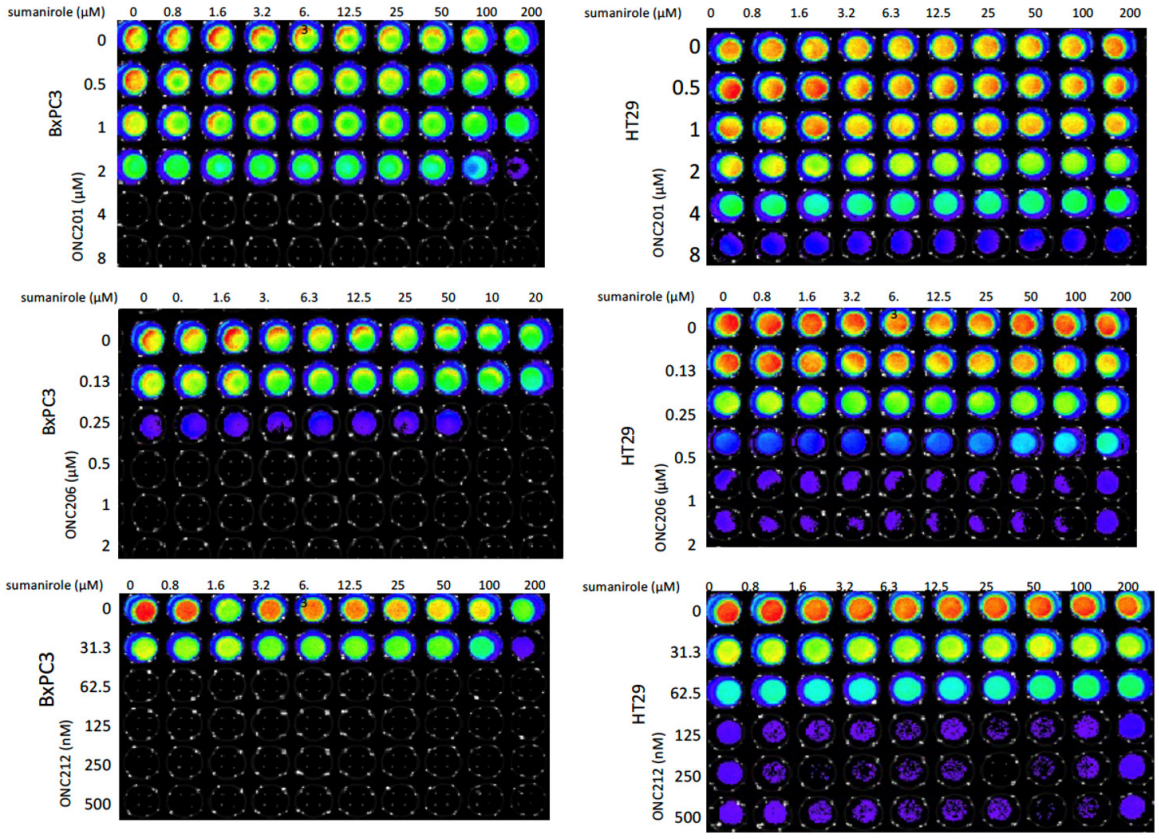

Figure 4. Impact of sumanirole pre-treatment on the cytotoxic effect of imipridone by CellTiter-Glo assay by CellTiter-Glo assay. CellTiter-Glo assay of the viability of BxPC3 and HT29 cell lines pre-treated with sumanirole for 48 H followed by sumanirole and ONC201/ONC206/ONC212 for 72 H is shown. Drug doses and cell lines are as indicated.

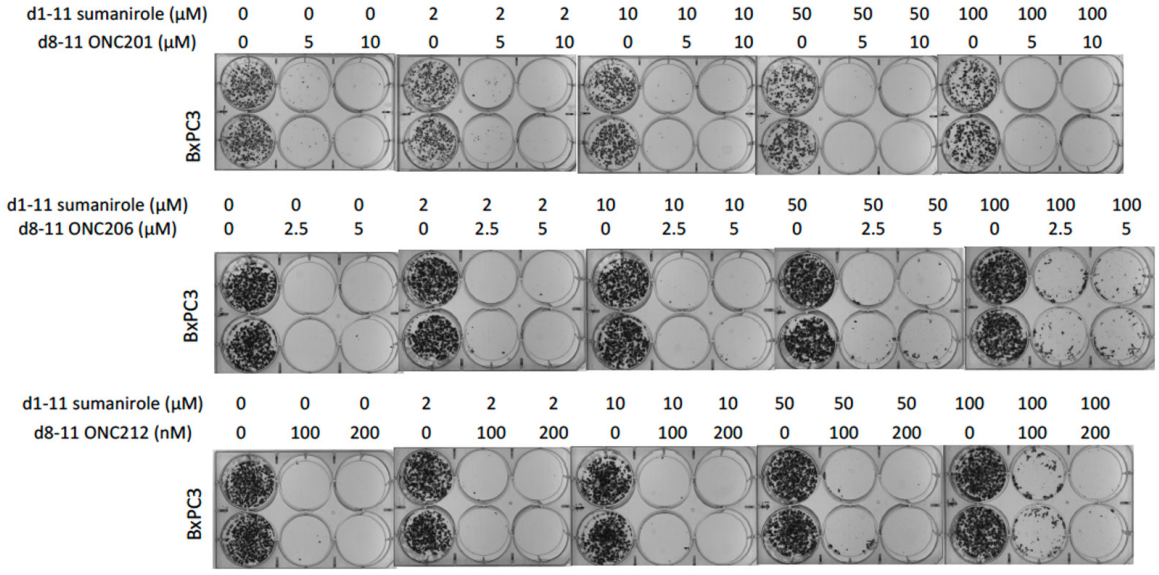

Figure 5. Impact of sumanirole pre-treatment on the cytotoxic effect of imipridone by colony assay. Colony assay of the viability of BxPC3 cell lines pre-treated with sumanirole for 7 days followed by sumanirole and ONC201/ONC206/ONC212 treatment are shown. Drug doses, treatment duration and cell lines are as indicated.

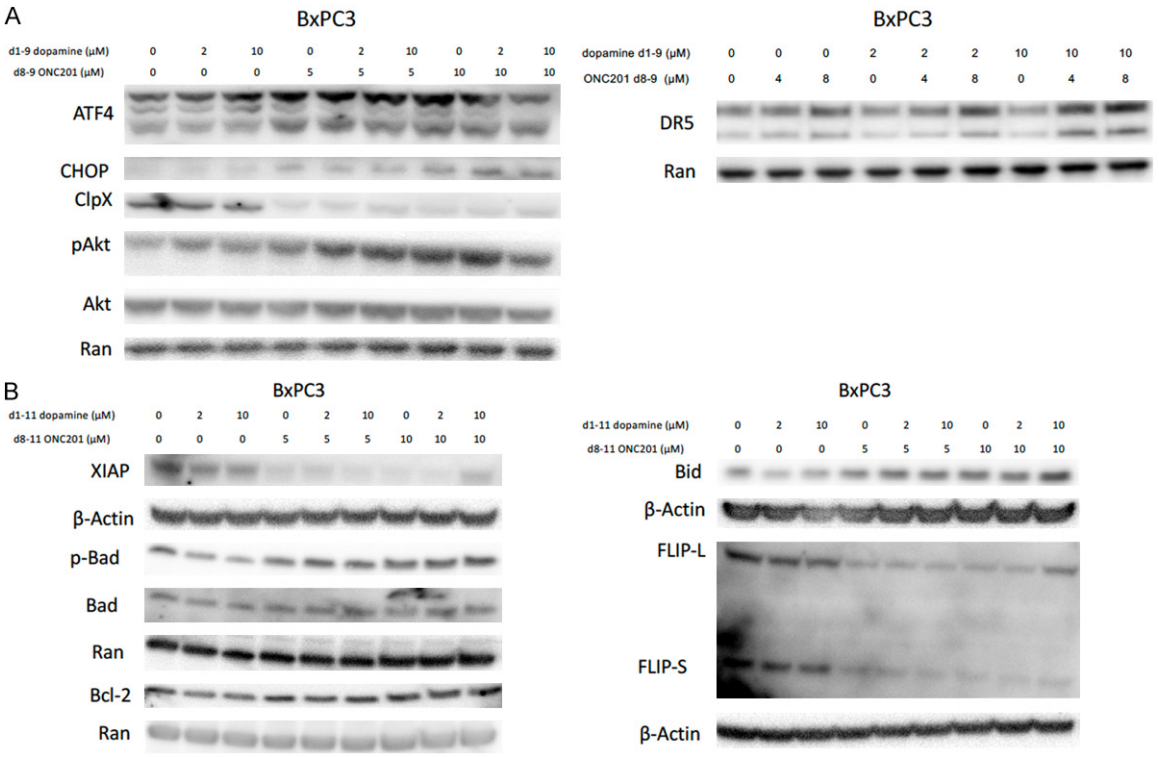

Figure 6. Impact of dopamine pre-treatment on the signaling pathway affected by ONC201 and the induction of anti- and pro-apoptotic proteins in BxPC3 cells. A. Impact of dopamine pre-treatment on the signaling pathway affected by ONC201 in BxPC3 cell line. B. Impact of dopamine pre-treatment on the anti- and pro-apoptotic proteins in the BxPC3 cell lines treated with ONC201.

increased and ClpX is reduced with ONC201, ONC206 or ONC212. We pretreated BxPC3 pancreatic cancer with dopamine for 7 days to induce adaptation to dopamine followed by treatment of ONC201 in the presence of continuous dopamine treatment for 24 hours and performed immunoblot for ATF4, CHOP, DR5 and ClpX. We did not observe any effect of dopamine pre-treatment on the induction of ATF4, CHOP or DR5 or reduction of ClpX caused by ONC201 (**Figure 6A**). Thus, the protection against the cytotoxic effect of ONC201 provided by dopamine pre-treatment is not due to the interference with imipridone engagement of the ISR or action on ClpX.

Dopamine pre-treatment up-regulates anti-apoptotic p-Bad, XIAP, c-FLIP and pAkt

To investigate other possible mechanisms of protection against the cytotoxic effect of ONC201 provided by dopamine pre-treatment of tumor cells, we pre-treated BxPC3 and HT29 cells with dopamine for 7 days followed by treatment with dopamine and ONC201/ONC206 for 72 hours in the presence of continuous dopamine treatment. We then performed an immunoblot for the pro- and anti-apoptotic proteins. X-linked inhibitor of apoptosis protein (XIAP) expression was reduced after treatment with ONC201 in BxPC3 cells (**Figure 6B**), and with ONC201/ONC206/ONC212 in HT29 cells (**Figure 7**) and pre-treatment with dopamine increased XIAP remarkably (**Figures 6B** and **7**).

It has been reported that XIAP abundance inversely correlates with sensitivity to ONC201-induced apoptosis and XIAP can prevent apoptosis in response to TRAIL induction [2]. Thus, XIAP induction may be involved in protection against the cytotoxic effect of ONC201 provided by dopamine pre-treatment. More pAkt was observed with dopamine pre-treatment in BxPC3 cells (**Figure 6A**), indicating a pro-survival effect and this can induce phosphorylation of Bcl-2-associated death promoter (BAD) that contributes to anti-apoptotic activity.

BAD inhibits antiapoptotic BCL-2, BCL-xL and BCL-W, and facilitates BAX/BAK activation in response to apoptotic stimuli [11, 12].

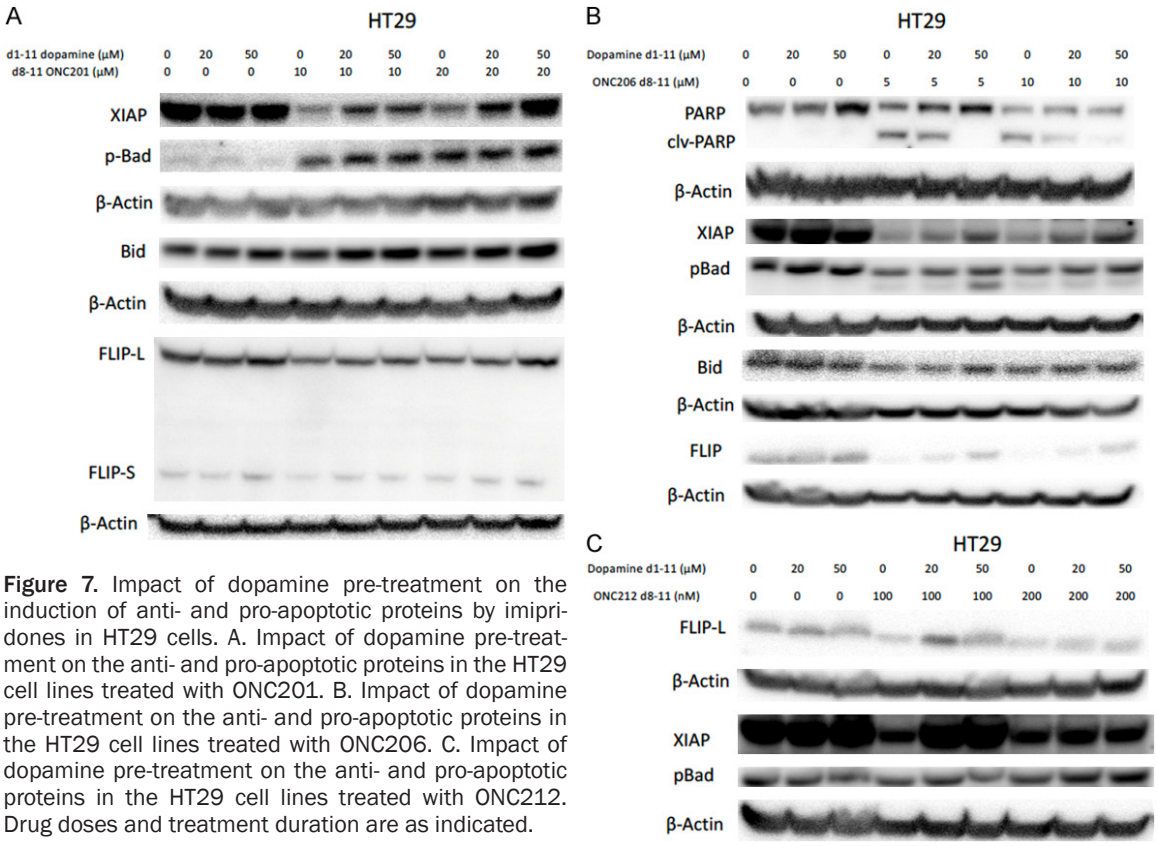

Figure 7. Impact of dopamine pre-treatment on the induction of anti- and pro-apoptotic proteins by imipridones in HT29 cells. A. Impact of dopamine pre-treatment on the anti- and pro-apoptotic proteins in the HT29 cell lines treated with ONC201. B. Impact of dopamine pre-treatment on the anti- and pro-apoptotic proteins in the HT29 cell lines treated with ONC206. C. Impact of dopamine pre-treatment on the anti- and pro-apoptotic proteins in the HT29 cell lines treated with ONC212. Drug doses and treatment duration are as indicated.

Phosphorylation prevents BAD from binding to anti-apoptotic proteins, and thus inactivates the pro-apoptotic activity of BAD [11, 13, 14]. We noted that adaptation to dopamine induced phosphorylation of BAD (**Figures 6B** and **7**), and thus plays an anti-apoptotic role that may contribute to the observed protection from cell death. c-FLIP, which inhibits caspase 8 activation and prevents cleavage of BID into tBID was upregulated with adaptation to dopamine (**Figures 6B** and **7**). Furthermore, BID was upregulated with adaptation to dopamine (**Figures 6B**, **7A** and **7B**), which implicates possibly less tBID and thus less pro-apoptotic activity. Therefore, the mechanism of the impairment of imipridone effect caused by adaptation to dopamine appears to involve upregulation of pAkt and anti-apoptotic p-Bad, XIAP, and c-FLIP.

Discussion

Binding and reporter assays have shown that ONC201 and ONC206 are selective antagonists of the dopamine D2-like receptors, specifically, DRD2 and DRD3 [5, 6]. This has prompted studies of the role of dopamine receptors in the mechanism of action of ONC201 and ONC206 in killing of tumor cells. In our previous studies, overexpression of DRD2 in tumor cells increased the cleavage of PARP induced by ONC201 but CRISPR-mediated knock-out DRD2 or DRD3 did not abrogate ONC201's effect on cell viability significantly [15]. A D2-receptor antagonist L741626 among other DRD2 antagonists was less potent at inducing both the ISR and apoptosis as compared to ONC201 [15].

We suspect that the action of ONC201 on some tumor cells may be attributed in part to DRD2. In our studies evaluating the synergy of HDACi and ONC201 in killing tumor cells, we attempted to modulate the expression of dopamine receptors on the tumor cells with histone deacetylase inhibitors (HDACi) in order to test the impact of modulation of dopamine receptors on the sensitivity to ONC201 [7]. We observed potent synergy with the combination of HDACi and ONC201, but the synergy was not correlated with the abundance of dopamine receptors as assessed by mRNA expression levels [7].

In the present studies, we hypothesized that dopamine, the agonist of DRD2, may compete with ONC201 or ONC206 for DRD2/3 and impair the anti-cancer effect of ONC201 or ONC206. We therefore treated tumor cells from different tissue origins with dopamine and ONC201 simultaneously and no impairment of ONC201's cytotoxic effect was observed. However, we observed that adaptation to dopamine protected some tumor cells from the cytotoxic effect of ONC201, ONC206 and unexpectedly, ONC212 as well, which was surprising as it has not been reported as a DRD2 antagonist.

Protection against the imipridones' cytotoxic effects provided by adaptation to dopamine showed heterogeneity among different types of cells. No protection was observed in tumor cells such as breast cancer (MCF7 and MDA-MB-468), gastric cancer (AGS) and diffuse midline gliomas (SU-DIPG-4 and SU-DIPG-36) cell lines. Protection was not observed by colony assays from BxPC3 cells treated with ONC212, though it was observed in CTG analysis. Because dopamine is not selective to DRD2, it is necessary to verify that the protection against the cytotoxic effect caused by adaptation of dopamine is caused by action on DRD2. We therefore pre-treated the tumor cells with the selective DRD2 agonist, sumanirole, followed by treatment with ONC201/ONC206/ONC212 in the presence of continuous sumanirole treatment, and the effect of the ONC201/ONC206/ONC212 on the tumor cells was not impaired by pre-treatment of sumanirole.

Our observations with the selective DRD2 antagonist sumanirole raised the question regarding whether DRD2 is involved in protection against imipridones' effect caused by adaptation to dopamine. In previous studies, transient knockdown of DRD2 reduced colorectal cancer cell number, at least in part, as a consequence of the activation of the integrated stress response [15]. In our present study, dopamine did not impact ISR or action of ONC201 on ClpP.

Previously, we demonstrated that XIAP abundance inversely correlates with sensitivity to ONC201-induced apoptosis and XIAP prevention of apoptosis in response to TRAIL induction [2]. In our present study, the mechanism of adaptation to dopamine impairing the imipridones' effect appeared to involve up-regulation of pAkt and anti-apoptotic p-Bad, XIAP, and c-FLIP. However, the up-stream signals of these anti-apoptotic proteins need to be further studied along with the role of dopamine receptors in the anti-apoptotic activity.

We suspect that dopamine as a small molecule may have effects on cells independent of its agonist activity towards dopamine receptors. We suspect that activation of cell survival signals rather than inhibition of the integrated stress response underlies protection by dopamine from imipridone-induced cell death. This includes ONC212 which is not known to antagonize dopamine receptors. Further studies are needed to unravel the connection between dopamine receptors and imipridone drug sensitivity as well as effect of dopamine on pro-survival pathways. The lack of protection of DMG cells raises further questions about the heterogeneity among tumor cell lines and the role of dopamine receptors in sensitivity of midline glioma to imipridones.

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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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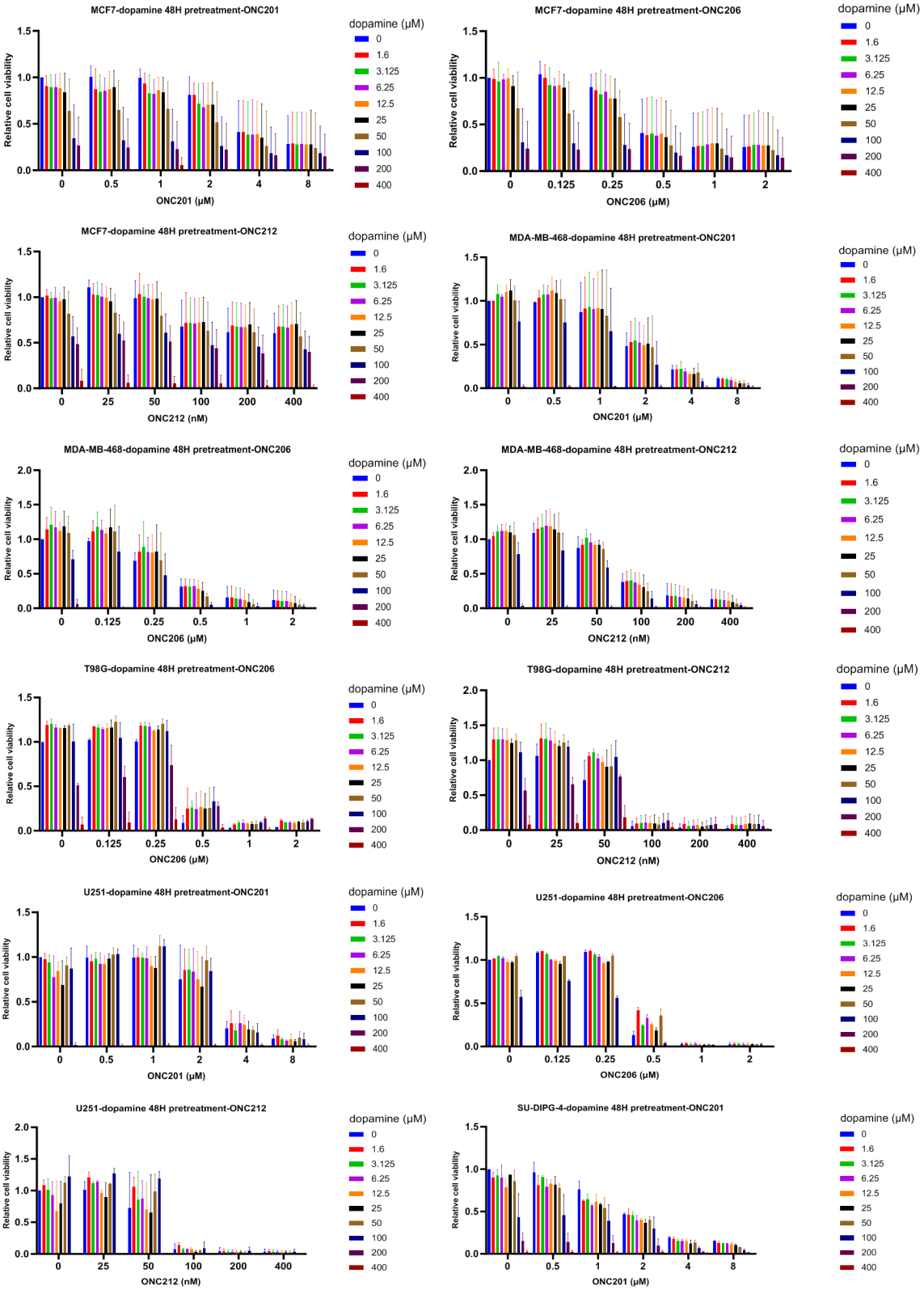
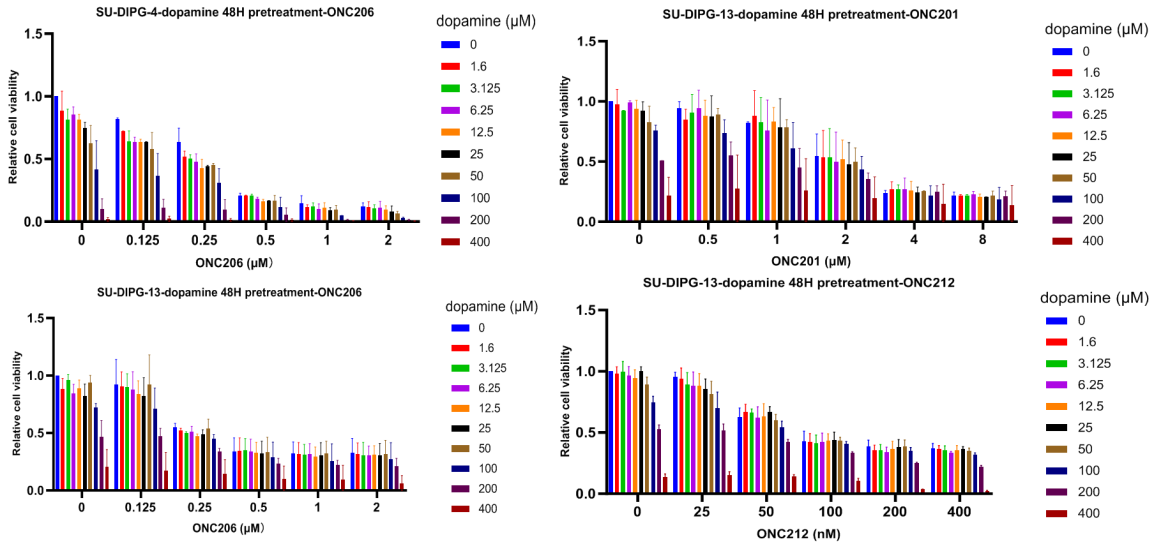
Dopamine pretreatment and imipridone-mediated cell death





Supplementary Figure 1. Impact of dopamine pre-treatment on the cytotoxic effect of imipridones by CellTiter-Glo assay. Cell Titer Glo assay of the viability of MCF7, MDA-MB-468, T98G, U251, SU-DIPG-4 and SU-DIPG-13 cells pre-treated with dopamine for 48 H followed by dopamine and ONC201/ONC206/ONC212 for 72 H is shown. Drug doses and cell lines are as indicated.