ONC212 is a novel mitocan acting synergistically with glycolysis inhibition in pancreatic cancer

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Abstract

ONC212 is a fluorinated-imipridone with preclinical efficacy against pancreatic and other malignancies. Although mitochondrial protease ClpP was identified as an ONC212-binding target, the mechanism leading to cancer cell death is incompletely understood. We investigated mitochondrial dysfunction and metabolic rewiring triggered by ONC212 in pancreatic cancer, a deadly malignancy with an urgent need for novel therapeutics. We found ClpP is expressed in pancreatic cancer cells and is required for ONC212 cytotoxicity. ClpX, the regulatory binding-partner of ClpP, is suppressed upon ONC212 treatment. Immunoblotting and extracellular flux analysis showed ONC212 impairs oxidative phosphorylation (OXPHOS) with decrease in mitochondrial-derived ATP production. Although collapse of mitochondrial function is observed across ONC212-treated cell lines, only OXPHOS-dependent cells undergo apoptosis. Cells relying on glycolysis undergo growth-arrest and upregulate glucose catabolism to prevent ERK1/2 inhibition and apoptosis. Glucose restriction or combination with glycolytic inhibitor 2-deoxy-D-glucose synergize with ONC212 and promote apoptosis in vitro and in vivo. Thus, ONC212 is a novel mitocan targeting oxidative-metabolism in pancreatic cancer, leading to different cellular outcomes based on divergent metabolic programs.
Introduction

ONC212 is a fluorinated imipridone demonstrating more potent anti-cancer activity in the nM range as compared to the first-in-class molecule ONC201 that is active in the low μM range (1, 2). Despite initially being discovered as TRAIL-inducing compounds (3), imipridones hinder tumor growth through a more complex, often cell context-dependent, mechanism (4). In solid cancers, they promote growth arrest and extrinsic apoptosis through the induction of the integrated stress response and upregulation of TRAIL/DR5 (5, 6). In addition, they act as synthetic ligands for specific G protein-coupled receptors often overexpressed on cancer cells, thus modulating cAMP-dependent signaling pathways that ultimately impact cell viability (7, 8). More recently, the mitochondrial peptidase ClpP has been identified as a novel intracellular target for both ONC201 and ONC212 in hematological cancers (9). ClpP is a serine protease located at the mitochondrial matrix where it is assembled into tetradecamers with ClpX, a nuclear-encoded chaperone working as ATP-dependent unfoldase and representing the regulatory subunit of the resulting ClpXP complex (10). Such complex plays fundamental roles in the mitochondrial unfolded protein response and is linked to the regulation of cellular bioenergetics (10). Imipridones dock directly into the hydrophobic pocket of the ClpP subunit, causing its enzymatic hyperactivation (9). Because many components of the respiratory chain complexes are putative ClpP substrates, ONC201 and ONC212 dysregulate the turnover of key enzymes involved in oxidative phosphorylation (OXPHOS), irreversibly undermining critical aspects of mitochondrial metabolism (9, 11).

Although historically recognized as a highly glycolytic tumor, pancreatic adenocarcinoma needs functional mitochondria to meet its energy demands (12–14). The oncogenic mitogen-activated protein kinase (MAPK) pathway, constitutively activated in pancreatic cancer, contributes to the regulation of mitochondrial dynamics, while mitochondrial respiration, in turn, provides the amount of adenosine triphosphate (ATP) needed to sustain cell growth and proliferation, especially under low glucose availability (12, 13). Even if a peculiar metabolic reliance may occur in specific cell types, OXPHOS and glycolysis usually cooperate to make cancer cells metabolically plastic, a kind of paradigm in cancer biology accounting for the failure of some targeted agents when administered as monotherapy (15).

Here, we examined the impact of ONC212 on the bioenergetic profile of pancreatic cancer cells, which broadly express its target ClpP. We found that mitochondrial metabolism is invariably suppressed upon ONC212 treatment, but the cellular outcomes actually differ based on divergent metabolic dependencies.

Materials and Methods
Cell cultures and reagents

All pancreatic cancer cell lines were purchased from American Type Culture Collection (ATCC). The cell lines were maintained in high glucose DMEM supplemented with glutamine 2 mM, pyruvate 1 mM, non-essential amino acids 1X (all from Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Cornig, NY, USA, Cat no. 30-002-CI) (complete medium, CM). In selected experiments, cells were cultured in no glucose DMEM (Thermo Fisher Scientific, Cat no. 11966025), supplemented with galactose 20 mM (Sigma Aldrich, St. Louis, MO, USA, Cat no. G5388), glutamine 2 mM, pyruvate 1 mM, non-essential amino acids 1X, 10% FBS and 1% penicillin-streptomycin. Cell lines were authenticated routinely and mycoplasma free. ONC212 was obtained from Oncoceutics, Inc (Philadelphia, PA, USA). MG132 was purchased from Selleckchem (Houston, TX, USA, Cat no. S2619), epoxomicin and bafilomycin A1 from Sigma-Aldrich (Cat no. E3652 and B1793, respectively). 2-deoxy-D-glucose (2-DG) was obtained from Sigma-Aldrich.

Cell viability assay and synergy assessment

Cell viability was measured by CellTiter-Glo (CTG, Promega, Madison, WI, Cat no. G7572). Briefly, 5x10^3 cells/well were seeded overnight in a 96-well plate at 37°C and 5% CO_2_. In selected experiments, the hypoxic environment (0.5–1% O_2_) was created using the INVIVO2 hypoxia workstation (TOUCAN Technologies). Cells were treated with either single agent or combination of ONC212 and 2-DG, at indicated concentrations. After 72 hours, luminescent-based cell viability was determined using CTG assay, following the manufacturer’s instructions. Percent of cell viability was determined by normalizing luminescence signal to control wells. Results were reported as % viability ± standard deviation (SD). Dose-response curves were generated and the half-maximal growth inhibition concentration (GI50) was calculated using GraphPad Prism version 7 (La Jolla, CA, USA). Compusyn software (ComboSyn, Inc.) was used to calculate the combination indices (CI). CI < 1 indicates some degree of synergy. CI < 0.5 indicates a very strong synergy.

siRNA transfection

Cells were plated in medium without antibiotics and incubated overnight. siRNA targeting ClpP (Santa Cruz Biotechnology, Dallas, TX, USA, Cat no. sc-60413) or AMPK (Santa Cruz Biotechnology, Cat no. sc-45312), or control siRNA (Quiagen, Hilden, DE, Cat no. 1022076) were transfected into cells using Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA, USA, Cat no. 13778075), following the manufacturer’s instructions. After 48 hours, cells were treated with vehicle (dimethyl sulfoxide, DMSO) or ONC212 for an additional 24 hours (WB...
experiments) or 72 hours (CTG experiments). Western blot was performed to assess the level of knockdown.

**Immunoblotting, extracellular lactate measurement, NAD\(^+\)/NADH determination, and immunofluorescence staining.**

See Supplementary Materials for additional details.

**Extracellular flux analysis**

5 x 10\(^4\) cells were seeded on XF96 cell culture microplates (Agilent Technologies Inc., Wilmington, DE, USA). After 24 hours, cells were treated with DMSO or ONC212 at the indicated concentrations for an additional 48 hours. Then the ATP Real-Time rate assay (Agilent Technologies Inc., Cat no. 103592-100) was performed on an Agilent Seahorse XFe96 analyzer, as described by the manufacturer. The ATP rate assay contains 10 mM of XF glucose, 1 mM of XF pyruvate, and 2 mM of XF glutamine. Serial injections of DMSO or ONC212 (port 1, final concentration 0.2 or 0.4 μM), oligomycin (port 2, final concentration 1.5 μM) and rotenone/antimycin A (Rot/AA, port 3, final concentration 0.5 μM) were applied. Oxygen consumption rate (OCR, pmol/min) and extracellular acidification rate (ECAR, mpH/min) were dynamically measured by the instrument. Proton efflux rate (PER, pmol H\(^+\)/min), OCR\(_{ATP}\) (i.e. the rate of oxygen consumption that is coupled to ATP production during OXPHOS), mitochondria-derived ATP production rate (mitoATP, pmol ATP/min), mitochondrial PER (mitoPER), glycolytic PER (glycoPER) and glycolysis-derived ATP production rate (glycoATP) were calculated as follows: PER = ECAR x buffer factor (mmol/L/pH) x Vol measurement chamber (μL) x scaling factor (Kvol); OCR\(_{ATP}\) = OCR - OCR\(_{Oligo}\); mitoATP = OCR\(_{ATP}\) x 2 (pmol O/pmol O\(_2\)) x 2.75; mitoPER = PER\(_{basal}\) - PER\(_{Rot/AA}\); glycoPER = PER - mitoPER; glycoATP = glycoPER. Data were processed with Wave software.

**In vivo studies**

The experimental *in vivo* protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University. Six- to seven-week old female nu/nu athymic mice were purchased from Taconic (Hudson, NY, USA). For the short-term experiment, 5 x 10\(^6\) BxPC3 or HPAF-II cells were suspended in 50 μL PBS mixed with 50 μL Matrigel (Thermo Fisher Scientific, Cat no. 354234), and injected subcutaneously into the rear flanks of 24 mice. Once tumor volume reached at least 100 mm\(^3\), mice were randomly assigned to one of four groups (3 mice/group): vehicle, ONC212, 2-DG, combination of ONC212 + 2-DG. ONC212 was delivered by oral gavage at the dosage of 50 mg/Kg in a solution of 70% PBS, 10% DMSO and 20% Kolliphor EL (Sigma Aldrich, Cat no. C5135), three times per week. 2-
DG was administered by intraperitoneal injection at the dosage of 500 mg/Kg diluted in PBS, three times per week. Mice were sacrificed after one week of treatment (= 3 doses) for biomarker analyses (immunohistochemistry (IHC) and blood tests). For the long-term experiment, 5 x 10^6 BxPC3 cells were injected in 24 mice following the same procedure as described above. Once tumor volume reached at least 100 mm^3, mice were randomly assigned to vehicle, ONC212, 2-DG, or the combination of ONC212 + 2-DG, administered three times per week at the same dosage as reported above. The treatment continued until mice developed signs of toxicity or discomfort from excessive tumor growth. Mice were weighed once a week to monitor signs of drug toxicity. The length (L) and width (W) of the masses were measured 3 times/week with a digital caliper, and the tumor volume was calculated applying the formula: 0.5*L*W^2. The analyses of tumor growth were limited to the mice starting treatment with a tumor volume of 100-250 mm^3 (vehicle = 4; ONC212 = 6; 2-DG = 4; combination = 5). Collection of whole blood and serum were performed by cardiac puncture and sent to Antech GLP (Morrisville, NC, USA) for blood cell count and chemistry tests. Tumors and organs were dissected and harvested for IHC.

**Immunohistochemistry**

Excised tumors were fixed in formalin. Paraffin embedding and sectioning of slides were performed by the Brown University Molecular Pathology Core Facility. Slides were de-waxed in xylene and subsequently hydrated in ethanol at decreasing concentrations. Antigen retrieval was carried out by boiling the slides in 2.1 g citric acid (pH 6) for 10 min. Endogenous peroxidase were quenched by incubating the slides in 3% hydrogen peroxide for 5 min. After nuclear membrane permeabilization with TBS-T, slides were blocked with horse serum (Vector Laboratories, San Francisco, CA, USA, Cat no. MP-7401-15), and incubated with primary antibodies overnight (Supplementary table S1) in a humidified chamber at 4°C. After washing with PBS, secondary antibody (Vector Laboratories, Cat no. MP-7401-15 or MP-7402) was added for 30 minutes, followed by DAB application (Thermo Fisher Scientific, Cat no. NC9276270), according to the manufacturer’s protocol. Samples were counterstained with haematoxylin, rinsed with distilled water, de-hydrated in an increasing gradient of ethanol, cleared with xylene, and mounted with Cytoseal mounting medium (Thermo Fisher Scientific, Cat no. 8312-4). Images were recorded on an Axioskop microscope (Zeiss), using QCapture. QuPath software was used to automatically count positive cells (16). For each IHC marker, 5 20x images per group were analyzed, and results were represented as the absolute number of positive cells per 20x field (17).

**Statistics**
Statistical analyses were conducted using GraphPad Prism 7. The Student’s two-tailed T test was used for pairwise analysis. Data are presented as means ± SD from at least 3 independent experiments, unless otherwise specified. Differences were considered significant for p values <0.05.

Results

The ClpXP complex is disrupted by ONC212 in pancreatic cancer cells

Although the ClpXP complex is often detected at the protein level in human cancers (10) and normal cells (Supplementary Fig. S1A), its expression in the specific context of pancreatic cancer has not been hitherto studied. Moreover, it is uncertain if the imipridone-induced ClpP hyperactivation, first described in blood cancers and a few solid tumor types (9, 18, 19), can be generalized to other malignancies. We analyzed ClpP and ClpX expression in a panel of pancreatic cancer cell lines, and assessed if ONC212 treatment could provoke ClpX suppression, a marker of uncontrolled ClpP activity (18, 20). We found that both ClpP and ClpX are highly expressed across all pancreatic cancer cell lines tested (Fig. 1A-D). ONC212 treatment, at doses comparable to the previously reported GI50 values (21), caused a slight, if any, reduction of ClpP protein level after 48 hours (Fig. 1A). Conversely, ClpX expression was abolished by the treatment (Fig. 1C, D). Kinetic analysis showed that ONC212 reduced ClpX expression in a time-dependent manner, with an almost complete suppression as soon as 6 hours after treatment (Supplementary Fig. S1B). Nude mice xenografted with BxPC3 or HPAF-II cells had reduced ClpX expression in the tumor mass after short-term treatment with ONC212 (Fig. 1E, F). Also human foreskin fibroblasts (HFF cell lines) had decreased ClpX expression in response to ONC212 (Supplementary Fig. S1A). In vitro co-treatment with the proteasome inhibitors MG132 or epoxomicin did not prevent ONC212-induced ClpX downregulation. The autophagy inhibitor bafilomycin A1 was not able to rescue ClpX expression either (Supplementary Fig. S1C). Instead, ClpP knockdown restored ClpX expression in cells treated with ONC212, suggesting ClpP is required for ONC212-triggered ClpX suppression (Fig. 1G). Therefore, ONC212 disrupted the ClpXP complex and rapidly promoted the acquisition of a ClpX<sup>neg</sup>ClpP<sup>pos</sup> phenotype, which is consistent with compounds that confer uncontrolled proteolytic capacity on ClpP (18, 20). To confirm that ClpP hyperactivation plays a role in the antitumor activity of ONC212 against pancreatic cancer, we performed knockdown experiments on multiple cell lines. As expected, ClpP downregulation by specific siRNA partially compromised the growth inhibitory effect of ONC212 in the Capan-2, HPAF-II and BxPC3 cells (Fig. 1H). Interestingly, in the absence of any treatment, the viability of ClpP-knockdown HPAF-II and BxPC3 cells was higher as compared to controls (Supplementary Fig. S2A). While this contrasts with data from acute
myeloid leukemia (AML) and prostate carcinoma, where ClpP knockdown impairs cell growth (10, 22), it is in keeping with pancreatic cancer TCGA transcriptomic results showing a positive correlation between ClpP expression and favorable prognosis (23).

ONC212 impairs mitochondrial bioenergetics

Several mitochondrial proteins involved inOXPHOS, Kreb’s cycle and mitochondrial translation have been identified as putative substrates of ClpP (9, 22). We focused on theOXPHOS machinery and addressed whether ONC212-induced ClpP hyperactivation could decrease the expression of key enzymes belonging to respiratory chain complexes. We found that NADH:ubiquinone oxidoreductase subunit A12 (NDUFA12, complex I), succinate dehydrogenase subunit A (SDH-A, complex II) and succinate dehydrogenase subunit B (SDH-B, complex II) were downregulated following 48 hours of treatment with ONC212 (Fig. 2A). This was consistent across all cancer cell lines, although the degree of protein downregulation was not identical, with HPAF-II and PANC-1 cells showing the most evident protein decrease. CFPAC-1 and Capan-2 cells were also tested at a shorter time point and showed similar protein suppression (Fig. 2B), suggesting that ONC212 affects the electron transport chain early on. Cytochrome C oxidase subunit IV (COXIV, belonging to complex IV) expression was also decreased by ONC212 in PANC-1 cells (Fig. 2A). ClpP knockdown partially prevented the downregulation ofOXPHOS proteins induced by ONC212 (Supplementary Fig. S2B). We next performed extracellular flux analysis to determine if OXPHOS enzyme downregulation might lead to mitochondrial functional impairment. ONC212 treatment decreased basal OCR in a dose-dependent fashion, uniformly across the cell lines tested (AsPC-1, BxPC3 and PANC-1) (Fig. 2C). As a consequence, mitochondria-derived ATP production rate (mitoATP) remarkably dropped with ONC212 treatment (Fig. 2C). The AMP-activated protein kinase (AMPK), a well-known molecular sensor of low energy status (24), is consequently phosphorylated at Thr172, with the exception of PANC-1 cells showing its constitutive activation (Supplementary Fig. S3A). Knockdown of AMPK decreased the viability of PANC-1 cells (Supplementary Fig. S3B, C) and enhanced their sensitivity to ONC212 (Supplementary Fig. S3D). Although AMPK activation might play a protective role in PANC-1 cells, the relevance of this mechanism appears cell-context dependent as AMPK knockdown in BxPC3 cells does not influence the sensitivity to ONC212 (Supplementary Fig. S3E).

Variable ONC212 sensitivity across pancreatic cancer cell lines

Given that OXPHOS inhibition occurred uniformly across all cancer cell lines upon ONC212 treatment, we wondered if its cytotoxic effect was similar among them. Cell viability assays performed after a 72-hour treatment period revealed that the cancer cell lines displayed
different sensitivities to ONC212, with GI50 values ranging from 0.09 to 0.47 μM. AsPC-1 and HPAF-II were the most sensitive, with GI50 of 0.09 and 0.11 μM, respectively (Fig. 3A). Although we used the ATP-based assay CTG to measure cell viability, which could have overestimated the anti-proliferative effect of ONC212, our results are comparable to those reported in our previous study where orthogonal methods were used (21). Importantly, AsPC-1 and HPAF-II underwent apoptosis upon treatment with 0.2 and 0.4 μM ONC212, as demonstrated by the induction of poly (ADP-ribose) polymerase (PARP) cleavage. By contrast, all other cell lines did not show any PARP cleavage in response to ONC212, indicating that, in some cells, the ONC212 effect was likely limited to growth inhibition, with no apoptotic triggering (Fig. 3B). Extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, a hallmark of pancreatic cancer due to constitutive Ras mutation (25) and a previously described imipridones’ downstream target (5), was affected similarly: while ONC212 inhibited ERK1/2 in the most sensitive cells, it turned out to be ineffective in the less sensitive ones (Fig. 3C). Therefore, ONC212 sensitivity paralleled ERK1/2 inhibition and PARP cleavage. Although ClpP expression and OXPHOS inhibition were shared across all ONC212-treated cell lines, some of them were eventually killed while others were just growth-arrested.

Glycolysis is boosted in ONC212-treated cell lines not undergoing apoptosis

Given the different cellular outcomes despite the same mitochondrial-impairing mechanism, we reasoned that cell-intrinsic metabolic features could tune the cytotoxic program of ONC212. Specifically, we focused on aerobic glycolysis, a major metabolic pathway supplying rapid ATP production and considered a possible escape mechanism from cancer therapeutics (26, 27). In BxPC3 and PANC-1 cells, representative of the ‘non-apoptotic’ subgroup, the glycolytic rate as measured by glycoPER was significantly upregulated in response to a 48-hour treatment with ONC212 (Fig. 4A). As a result, the glycolysis-derived ATP production rate (glycoATP) increased and contributed to maintaining an acceptable amount of total ATP (Fig. 4B). Also, oligomycin injection did not further increase the glycolytic rate in ONC212-treated cells, suggesting the cell glycolytic capacity already reached its maximum (Supplementary Fig. S4A). In stark contrast, AsPC1 cells, which usually die by apoptosis under ONC212, failed to sustain glycolysis and their glycoPER dramatically dropped, thus suffering a profound decline in ATP production (Fig. 4A, B). The upregulation of the glycolytic pathway in the less sensitive cell lines was further supported by the increased extracellular lactate concentration observed after ONC212 treatment in a time- and dose-dependent manner (Fig. 4C). Furthermore, in BxPC3 cells ONC212 perturbed the NAD+/NADH ratio in favor of NAD+, an essential cofactor fueling the glycolytic flux (Fig. 4D). In search of enzymes possibly involved in the glycolytic switch, we found that hexokinase II
(HK II), catalyzing the phosphorylation of glucose to glucose-6-phosphate, was upregulated in response to ONC212 both in BxPC3 and PANC-1 cells. The glucose transporter GLUT1 increased in BxPC3 cells after treatment, while PANC-1 did not express it. Lactate dehydrogenase A (LDHA) was expressed in both cell lines without significant changes after treatment (Supplementary Fig. S4B).

Collectively, ONC212 affected the metabolic profiles in a cell-context dependent manner. BxPC3 and PANC-1 cells, typically resistant to ONC212-induced apoptosis, became less oxidative but highly glycolytic. On the contrary, bioenergetics of the most sensitive AsPC-1 cells was completely turned off by the treatment, due to their inability to sustain glycolysis (Fig. 4E).

**Basal mitoATP% may be a new functional biomarker of ONC212 sensitivity**

Considering aerobic glycolysis as an oncogenic metabolic pathway potentially counteracting the antineoplastic activity of ONC212, we examined the basal metabolic profile of different cancer cell lines to find possible correlations with ONC212 sensitivity. We discovered that AsPC-1 and HPAF-II cells, displaying the lowest GI50 upon ONC212 treatment as well as ERK1/2 inhibition and PARP cleavage, were predominantly oxidative, as the percentage of mitochondria-derived ATP production (mitoATP% = mitoATP production rate/total ATP production rate * 100) was 56.0% ± 7.7 and 59.5% ± 3.7, respectively. Conversely, the less sensitive BxPC3 and PANC-1 cells were significantly more glycolytic, with mitoATP% far below 50% (38.2% ± 1.3 and 34.3% ± 7.7, respectively). Therefore, basal mitoATP% could represent a novel, metabolism-based, functional biomarker positively correlating with ONC212 sensitivity (Fig. 5A, B). To further investigate if the cellular metabolic background influenced ONC212 sensitivity, we cultured the less sensitive cell lines (BxPC3, PANC-1 and Capan-2) in glucose-free DMEM containing galactose, which is a well-established method to force cells to use OXPHOS instead of glycolysis to get ATP (28). As shown in Fig. 5C, galactose-cultured cells were more susceptible to ONC212 activity compared to those cultured in standard glucose-containing medium. On the other hand, culturing the most sensitive cells (AsPC-1 and HPAF-II) under hypoxia, which favors a metabolic switch towards glycolysis (29), rendered them more resistant to ONC212 (Fig. 5D). Altogether, these findings suggest that the basal metabolic profile, which is highly dynamic in nature and affected by both cell-intrinsic and environment-based mechanisms, may dictate cell sensitivity to ONC212.

**Glycolysis inhibition converts the cytostatic effect of ONC212 into a pro-apoptotic one**
We next evaluated whether ONC212 could trigger cell death in BxPC3 and PANC-1 cells when combined with glycolysis suppression. While cells cultured in glucose were able to sustain ERK1/2 phosphorylation in the presence of ONC212, those grown up in galactose underwent ERK1/2 inhibition after 48-hours of treatment (Fig. 5E). In addition, analysis of PARP cleavage demonstrated that ONC212 could trigger apoptosis when cells were cultured in galactose, while ineffective in apoptosis induction under glucose-containing conditions (Fig. 5E). To further investigate if the simultaneous inhibition of glycolysis could deepen the cytotoxic activity of ONC212, PANC-1 and BxPC3 cells were treated with ONC212 combined with 2-DG, a well-characterized glycolysis inhibitor acting on the first steps of this pathway (30). We found that the combination was highly synergistic in both cell lines, with CI far below 0.5 for various combinatorial doses (Fig. 6A). Importantly, combination treatment, but not ONC212 or 2-DG alone, inhibited ERK1/2 phosphorylation and induced PARP cleavage (Fig. 6B). Therefore, glycolysis sustained the MAPK pathway and promoted apoptotic evasion in the presence of ONC212. In glycolysis-addicted cell lines, shut down of glucose catabolism was needed to turn the cytostatic effect of ONC212 into a pro-apoptotic one (Fig. 6C).

**ONC212 synergizes with 2-DG in vivo**

To test if the combination of ONC212 and 2-DG could be effective in vivo, we generated a xenograft model of pancreatic cancer using the ONC212-resistant BxPC3 cell line. BxPC3 cells were chosen over the PANC1 due to their higher tumor-forming capacity in vivo (21). Once the tumor volume reached at least 100 mm³, mice were randomly assigned to the treatment with vehicle, ONC212, 2-DG, or the combination. Unlike ONC212 and 2-DG alone, the combination of both agents halted tumor growth in comparison with the control group (P=0.0082 at 30 days after treatment start) (Fig. 6D, Supplementary Fig. S5A). Moreover, the combination remarkably reduced the variability of tumor growth among mice within the same group, suggesting that simultaneous inhibition of OXPHOS and glycolysis might prevent the metabolic escape from each individual blockade (Supplementary Fig. S5A). IHC analysis showed the combination, but not single agents, impaired tumor cell proliferation and increased apoptosis as early as one week after treatment initiation. ERK phosphorylation was also significantly inhibited in vivo by ONC212 when combined with 2-DG, whereas the single-agent treatments resulted ineffective (Fig. 6E, Supplementary Fig. S5B). Blood cell count revealed leukocytopenia in the combination group (Supplementary Table S2), as a sign of potential bone marrow toxicity selectively involving the white series. Serum chemistry panel showed normal kidney and liver function, with the exception of a mild reduction of total proteins and albumins across all groups, likely due to the tumor itself (Supplementary Table S3). Also, histological assessment of liver sections did not show any obvious sign of toxicity (Supplementary Fig. S6A). Monitoring of body weight showed no significant decrease in the...
combination group, whereas one and two mice in the control and ONC212 groups, respectively, were sacrificed during treatment because of >15% weight loss (Supplementary Fig. S6B).

Altogether, ONC212 and 2-DG might be an effective and well-tolerated drug combination to target complementary, pro-survival metabolic pathways in pancreatic cancer.

Discussion

With an estimated 5-year survival rate of 9.3%, pancreatic cancer is one of the most lethal human tumors. Standard pharmacological treatment is still based on toxic, yet often unsuccessful, combination chemotherapy with a dearth of effective targeted agents (31). The fluorinated imipridone ONC212 has been recently identified as a novel candidate in pancreatic cancer therapeutics (21). However, the way it counteracts tumor growth, the determinants of its antineoplastic activity and the strategies implemented by cancer cells to survive are not well defined. Our study provides evidence that ONC212 impacts mitochondrial functions and rewire the metabolism of pancreatic cancer cells, acting, de facto, as a novel mitocan (32). In particular, ONC212 a) disrupts ClpXP complex causing profound downregulation of the regulatory subunit ClpX, while leaving unaffected the catalytic subunit ClpP, which is required for its anticancer activity, b) hinders OXPHOS by suppressing critical respiratory chain complex components, c) exerts its cytotoxic effects in a cell- and environment-context dependent manner, with glycolysis emerging as an oncogenic escape metabolic pathway negatively regulating ONC212 sensitivity in vitro and in vivo.

ClpP, the recently discovered target of imipridones, is an evolutionarily ancient mitochondrial protease often overexpressed in cancer cells (9, 10). Pancreatic cancer cells also broadly express ClpP at the protein level, but its genetic knockdown does not significantly impair cell viability. This contrasts with other cancers such as AML, where ClpP is necessary to maintain cell growth (22). Because ClpP knockdown suppresses mitochondrial metabolism (10), the ability of cancer cells to cope with mitochondrial dysfunction may guide their outcome upon ClpP genetic ablation. The ClpP-binding partner ClpX dramatically decreases upon ONC212 treatment, consistent with previous studies showing that different ClpP-activating compounds displace ClpX and lead to its degradation through yet poorly defined mechanisms (18, 20). Knockdown experiments indicate ClpP is required for ONC212-triggered ClpX suppression. Although this may suggest that aberrantly active ClpP could rapidly degrade its own regulatory subunit, previously reported cell-free assays have
documented that ClpX, unlike casein, is not directly degraded by purified, ONC212-treated ClpP (9). Because we show that cytoplasmic proteasomal and lysosomal degradation plays no role in ClpX downregulation, it is possible that additional proteases residing in the mitochondrial matrix, such as Lon, might be involved in ClpX degradation upon ONC212 exposure. Whether the downregulation of ClpX is critical for ONC212 activity or only represents an epiphenomenon of uncontrolled mitochondrial proteolysis will also need to be clarified in future studies involving ClpX-overexpressing models.

ClpXP dysregulation affects mitochondrial functions. Using immunoblotting and live cell-based metabolic analysis as two complementary approaches, we demonstrated that ONC212 reduces the expression of multiple components of the electron transport chain ultimately leading to OXPHOS collapse. The resulting energetic stress leads to AMPK phosphorylation, a signaling pathway that mammalian cells often use to restore energy imbalance and promote survival (24, 33). Accordingly, AMPK knockdown increases sensitivity to ONC212 at least in PANC-1 cells. This may have interesting translational implications as LKB1, the kinase upstream to AMPK and responsible for its activation, is deleted or mutated in a subset of human cancers showing remarkable sensitivity to energy-stress inducing agents (34). Thus, ONC212 may be particularly powerful against LKB1-disrupted tumors, lacking the ability to cope with energetic stress.

Despite our focus on OXPHOS enzymes, the protease activity of the imipridones’ target, whose substrates cover a wide spectrum of proteins involved in several aspects of mitochondrial pathophysiology (9, 22), provides the opportunity to perturb different, mitochondria-centered, homeostatic pathways simultaneously. This may explain why ONC201 and ONC212 have, at least in vitro, broad anticancer activity (4) as opposed to most targeted therapies, which usually affect one specific signaling pathway that a particular type of cancer may be addicted to (35). Using functional metabolic approaches, we found that OXPHOS dependency positively correlates with the depth of response to ONC212. The OXPHOS-addicted cell lines AsPC-1 and HPAF-II have the lowest GI50 and undergo apoptosis upon ONC212 treatment, whereas the glycolysis-addicted BxPC3 and PANC-1 cells undergo growth arrest without apoptosis induction. Essentially, the more oxidative the cancer cells are, the higher their chance of being killed by ONC212. Should our findings be confirmed in larger panels of cell lines and organoids, we may hypothesize to test primary cancer cells or patient-derived organoids for mitoATP production, thus predicting imipridone sensitivity based on tumor-specific metabolic dependence. Future investigations about how ONC212 affects the assembly of respiratory complexes, and how complex-specific mitochondrial respirations are impacted by ONC212 treatment, will better define the
metabolic dependencies specifically targeted by this drug. Dihydroorotate dehydrogenase-dependent respiration, which is essential to drive pyrimidine biosynthesis, may be also affected by ONC212 as it shows broad anti-proliferative activity (36). Alternatively to functional metabolic aspects, molecular biomarkers potentially correlating with metabolic signatures could represent a valuable strategy to guide treatment with imipridones in pancreatic cancer. Primary pancreatic tumors and cell lines might express different levels of OXPHOS proteins, indicating different susceptibility to mitochondria-targeting agents. The fact we evaluated only some of the OXPHOS machinery members represents a limitation of our study, which does not permit definitive correlations between the level of OXPHOS proteins and the sensitivity to ONC212. Moreover, in our study the amount of ClpP modulates ONC212 in vitro efficacy, suggesting that ClpP levels in primary tumors may predict patients’ response to ONC212 treatment.

Our findings in pancreatic cancer cells are in agreement with recent reports highlighting the importance of tumor oxidative metabolism in addition to aerobic glycolysis (12–14), a concept that downsizes Warburg’s effect and points to the coexistence of different energy-producing pathways working together and sometimes substituting for each other (15). In our study, glycolysis-addicted cells further increase their glycolytic flux under ONC212. Interestingly, an activator of fungal ClpP termed dioctatin also inhibits oxidative metabolism and enhances A. flavus glycolysis and alcohol fermentation, hinting that upregulation of glucose catabolism may be an evolutionarily conserved response to ClpXP perturbation (37). Such metabolic remodeling sustains ATP production, which is needed, in turn, to keep ERK phosphorylation sufficiently high and avoid cell death. In this regard, our results highlight two basic concepts. First, ERK status under ONC212 is not driven by ONC212 per se, but rather by the cancer cell response to ONC212. Therefore, despite initially described as dual AKT/ERK inhibitors (5), imipridones likely affect these pathways in a cell-context dependent manner. Second, there is bidirectional interplay between the oncogenic Ras-MEK-ERK pathway and glucose metabolism. While KRAS activation drives glycolysis (38), glucose catabolism enhances MAPK signaling, likely supplying ATP and intermediate metabolites fueling the oncogenic cascade (39). Such a compounding cycle can be effectively blocked by the combination of ONC212 and 2-DG, simultaneously inhibiting parallel bioenergetic pathways. Our in vivo experiments show this combination is effective in an ONC212-resistant tumor model and devoid of relevant toxicity. This suggests that energy deprivation due to dual inhibition of OXPHOS and glycolysis does have a therapeutic window and proves more lethal in the context of oncogene-driven anabolism as compared to non-transformed cells, which enter a quiescent state allowing them to survive nutrient stress (40). Beside 2-DG, other glycolytic inhibitors may be investigated as potential candidates for combination with ONC212. Among
them, LDHA inhibitors could be particularly promising due to their ability to block pyruvate-to-lactate conversion and NAD\(^+\) regeneration, which is essential to fuel the glycolytic flux \((41)\).

Furthermore, the combination of ONC212 and clinically-approved MEK inhibitors may be synergistic due to the increased OXPHOS dependency conferred by MAPK inhibition \((14)\).

In addition to cell-intrinsic metabolic background, the surrounding microenvironment, in terms of oxygen and nutrients availability, can influence ONC212 sensitivity. In our study, high levels of glucose and hypoxic environment emerged as extracellular factors negatively impacting ONC212 efficacy. As pancreatic cancer displays intra-tumoral heterogeneity due to different amounts of nutrient and oxygen supply in different neoplastic areas \((42)\), we speculate that cancer cells within the same tumor may respond differently to ONC212 based on their specific localization. Additional experiments investigating ERK status and apoptotic markers in ONC212-treated cells under normoxia and hypoxia will be required to corroborate our findings.

In conclusion, ONC212 is a novel mitocan acting on the ClpXP complex and causing collapse of mitochondrial bioenergetics. While all pancreatic cancer cells express the target ClpP, only some of them functionally rely on mitochondrial metabolism and are effectively killed by ONC212. Though acting as an escape mechanism in some cell types or specific environmental contexts, glycolysis emerges as a metabolic vulnerability exposed by ONC212 and potentially suitable for strategic drug combinations.

**Acknowledgements**

This work was presented in part at the 2020 American Association for Cancer Research (AACR) meeting. The work was supported by a Warren Alpert Foundation grant (to W.S.E-D.), an NIH grant to W.S.E-D. (CA173453) and the Mencoff Family Professorship at Brown University (W.S.E-D.). W.S.E-D. is an American Cancer Society Research Professor.

**Author Contributions**

I.F. and W.S.E-D. conceptualized and designed the experiments. I.F. performed experiments with some assistance from L.Z. and A.L. All authors contributed to the interpretation of the results and the writing of the manuscript. W.S.E-D. provided resources and overall oversight of the research.


17. Pingping Hou, Avnish Kapoor, Qiang Zhang, Jiexi Li, Chang-Jiu Wu, Jun Li, et al.


23. https://www.proteinatlas.org/ENSG00000125656-CLPP/pathology


Figure Legends

Fig 1. ONC212 alters ClpXP expression and inhibits pancreatic cancer cell viability via ClpP. (A) Western blot analysis for ClpP was performed in the indicated pancreatic cancer cell lines treated or not with ONC212 for 48 hours. (B) Immunofluorescence detection of ClpP (Cy3) in AsPC1 cells, showing its co-localization with the mitochondrial marker TOM20 (Alexa Fluor 488). Sample was counterstained with DAPI and visualized by confocal microscopy. Magnification 63x. Scale bar: 6 μm. (C) Immunofluorescence analysis of ClpX (Alexa Fluor 488) in BxPC3 cells treated or not with 0.2 μM ONC212 for 24 hours. Magnification: 100x. Scale bar: 9 μm. (D) Western blot analysis for ClpX in the indicated cell lines treated or not with ONC212 for 48h. (E) IHC staining of ClpX in BxPC3 xenografts treated with 3 doses of vehicle or ONC212. Images were captured at 20x magnification. Scale bar: 100 μm. Data are expressed as mean ± SEM. ***p<0.001 (F) IHC staining of ClpX in HPAF-II xenografts treated with 3 doses of vehicle or ONC212. Images were captured at 20x magnification. Scale bar: 100 μm. Data are expressed as mean ± SEM. ***p<0.001 (G) The indicated cell lines were transfected with scrambled siRNA ClpP-specific siRNA (siClpP). After 24 hours, cells were treated with 0.2 μM ONC212 for an additional 24 hours. Western blot analysis for ClpP and ClpX was performed. (H) The indicated cell lines were transfected with scrambled siRNA (siControl) or ClpP-specific siRNA (siClpP). After 48 hours, cells were treated with ONC212 for an additional 72 hours. Afterwards, cell viability was assessed by CTG assay. Dose-response curves summarizing results from three independent experiments are shown. Blots in (A), (D) and (G), and images in (B) and (C) are representative of three independent experiments.

Fig 2. ONC212 inhibits OXPHOS. (A) Western blot analysis for NDUFA12, SDH-A, SDH-B and COXIV in the indicated cell lines treated or not with ONC212 for 48 hours. (B) Western blot for NDUFA12, SDH-A and SDH-B in CFPAC-1 and Capan-2 cells treated with the indicated doses of ONC212 for 24 hours. Blots in (A) and (B) are representative of three independent experiments. (C) AsPC-1, BxPC3 and PANC-1 cells were treated for 48 hours with 0.2 or 0.4 μM ONC212 (ONC) prior to performing the ATP Real-Time rate assay. Continuous extracellular flux analysis was conducted to record basal OCR (upper panels) and calculate basal mitochondrial ATP production rate (mitoATP) (lower panels). Data are expressed as mean ± SD. *p<0.05, ***p<0.001.
Fig 3. The antineoplastic effect of ONC212 is cell-context dependent. (A) The indicated cancer cell lines were treated with ONC212 for 72 hours. Cell viability was assessed by CTG assay. Dose-response curves are shown and GI50 values are summarized according to ONC212 sensitivity. (B) Western blot analysis for PARP cleavage in the indicated cell lines treated or not with ONC212 for 48 hours. (C) Western blot analysis for ERK phosphorylation in the indicated cell lines treated or not with ONC212 for 48 hours. Blots are representative of at least three independent experiments.

Fig 4. Glycolysis modulation in response to ONC212. (A-B) AsPC-1, BxPC3 and PANC-1 cells were treated for 48 hours with 0.2 or 0.4 μM ONC212 (ONC) prior to performing the ATP Real-Time rate assay. (A) Dot plots showing the glycolytic rate, as measured by glycoPER. (B) Bar charts showing the glycolysis-derived (glycoATP) and mitochondria-derived (mitoATP) ATP production rate (upper panel). The percentage of ATP generated from each pathway is plotted in the lower panel. Means and SD are represented. N = 4 independent experiments. *p<0.05, **p<0.01, ***p<0.001. (C) The indicated cell lines were treated with either vehicle or ONC212 at the indicated doses. Lactate RLU was measured after 24 or 48 hours in culture supernatants and normalized to cell viability (expressed in RLU) as measured by CTG assay. N = 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001. (D) BxPC3 cells treated or not with ONC212 for 24 hours were permeabilized and the levels of NAD+ and NADH were individually assessed. NAD+/NADH ratio is plotted. N = 4 independent experiments. Means and SD are represented. ***p<0.001. (E) Energetic maps showing mitoATP production rate and glycoATP production rate of BxPC3, PANC-1 and AsPC-1 cells treated with increasing concentrations of ONC212. Mean and SD of four independent experiments are shown.

Fig 5. OXPHOS dependence predicts ONC212 sensitivity. (A-B) HPAF-II, AsPC-1, BxPC3 and PANC-1 cells were seeded in a 96-well Seahorse plate at a density of 30000 cells/well. After 48 hours, when cells reached a confluence of about 80 to 90%, the ATP Real-Time rate assay was performed. (A) Percentages of mitoATP and glycoATP are shown for each cell line. N = 3 independent experiments. **p<0.01. (B) Energetic maps showing the basal metabolic profile of the indicated cell lines. Shown are mean and SD of three independent experiments. (C) BxPC3, PANC-1 and Capan-2 cells were cultured in glucose- or galactose-containing medium and treated with increasing concentrations of ONC212 for 72 hours. Cell viability was assessed by CTG assay. Dose-response curves summarizing results
from three independent experiments are shown. (D) HPAF-II and AsPC-1 were treated with increasing concentrations of ONC212 in either normoxia or hypoxia for 72 hours. Cell viability was assessed by CTG assay. Dose-response curves summarizing results from three independent experiments are shown. (E) BxPC3 and PANC-1 cells were cultured in glucose- or galactose-containing medium and treated with ONC212 at the indicated concentrations for 48 hours. At the end of culture, western blot analysis for ERK phosphorylation and PARP cleavage was performed.

**Fig 6.** ONC212 synergizes with the glycolysis inhibitor 2-DG. (A) BxPC3 and PANC-1 cells were treated with the combination of ONC212 (0.06-2 μM) and 2-DG (0.19-12.5 mM) for 72 hours. Cell viability was assessed by CTG assay and CI was calculated for each dose combination using Compusyn software. As evidenced by the heat maps (*left panels*), CI is less than 0.5 for most dose combinations, indicating a strong synergism. Graphs (*right panels*) represent a selected synergistic dose of ONC212 and 2-DG, with the corresponding CTG image and CI. (B) Western blot analysis of PARP cleavage and ERK phosphorylation in PANC-1 cells treated for 48 hours with vehicle, 0.4 μM ONC212, 25 mM 2-DG or the combination of the two agents. (C) Model for conversion of drug-resistant, glycolysis-addicted tumors to apoptotic phenotypes by combining ONC212 with a glycolysis inhibitor, such as 2-DG. (D) Nude mice xenografted with BxPC3 cells were treated 3 times/week with vehicle, ONC212, 2-DG, or the combination of both agents. Represented are the tumor volumes measured 30 days after treatment initiation. At this time point, 1 mouse in the ONC212 group and 1 mouse in the 2-DG group had already been sacrificed due to low body weight and excessive tumor growth, respectively. Data are expressed as mean ± SEM. (E) IHC staining of ki67 (proliferation marker), cleaved caspase 3 (cc3, apoptosis marker) and pERK in BxPC3 xenografts treated with 3 doses of the indicated agents. Images were captured at 20x magnification. Scale bar: 100 μm. Data are expressed as mean ± SEM. **p<0.01, ***p<0.001.
Figure 1

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B

Image B. DAPI, TOM20, ClpP, and merge images for different cell lines.

C

Image C. ClpX/DAPI images for different conditions.

D

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E

Image E. ClpX cell number per 20x view for different conditions.

F

Image F. ClpX images and graph showing cell number per 20x view.

G

Table G. siRNA ClpP experiment results for different cell lines.

H

Graphs H. Normalized cell viability for different cell lines with siControl and siClnP conditions.
Figure 2

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| mitoATP (pmol/min) |        |        |
| vehicle |        |        |
| ONC 0.2 |        |        |
| ONC 0.4 |        |        |
| ** *** |        |        |
| ** *** |        |        |
| ** *** |        |        |

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Figure 4

A

BxPC3  |  PANC-1  |  AsPC-1

glycoPER (pmol/min)

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***  |  ***

B

BxPC3  |  PANC-1  |  AsPC-1

ATP (pmol/min)

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***  |  ***

ATP%

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***  |  ***

C

BxPC3  |  PANC-1  |  Capan-2

Normalized Lactate RLU

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***

D

BxPC3

NAD+/NADH

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***

E

BxPC3  |  PANC-1  |  AsPC-1

glycoATP (pmol/min)

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***

mitoATP (pmol/min)

vehicle  |  ONC 0.2  |  ONC 0.4

**  |  *  |  ***
Figure 5

A

![Bar graph showing ATP levels in HPAF-II, AsPC-1, BxPC3, and PANC-1 cells. The graph compares glycoATP and mitoATP levels with error bars.](image)

B

![Scatter plot showing the correlation between glycoATP and mitoATP levels in cells undergoing apoptosis and cells not undergoing apoptosis. The plot includes data points for HPAF-II, AsPC-1, BxPC3, and PANC-1 cells.](image)

C

![Graphs showing normalized cell viability for BxPC3, PANC-1, and Capan-2 cells treated with ONC212 at different concentrations of glucose and galactose.](image)

D

![Graphs showing normalized cell viability for HPAF-II and AsPC-1 cells under normoxic and hypoxic conditions.](image)

E

![Table showing normalized cell viability for BxPC3 and PANC-1 cells treated with ONC212 at different concentrations of glucose and galactose.](image)
Figure 6

A

Synergy ■ Antagonism

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ONC212 μM

Normalized cell viability

CI = 0.12

CI = 0.14

B

ONC212 - + + +
2-DG - - + +

PARP       97 kDa
cPARP      51 kDa
pERK       51 kDa
ERK        51 kDa
Ran        28 kDa

C

Degradation of respiratory chain enzymes

oxphos inhibition

mitochondria

oxphos addicted

glyco addicted

Decreased ATP
Activation of AMPK
ERK inhibition
Apoptosis

Decreased ATP
Activation of AMPK
Glycolysis
ERK phosphorylation
Drug resistance

D

P = 0.0082

Tumor volume (mm²)

Vehicle ONC212 2-DG Combination

E

Vehicle ONC212 2-DG Combination

ki67

cc3

pERK
Molecular Cancer Therapeutics

ONC212 is a novel mitocan acting synergistically with glycolysis inhibition in pancreatic cancer

Isacco Ferrarini, Anna Louie, Lanlan Zhou, et al.

Mol Cancer Ther Published OnlineFirst June 17, 2021.

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