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Repurposing Metformin, Simvastatin and Digoxin as a combination for Targeted therapy of Pancreatic Ductal Adenocarcinoma

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Abstract

Patients with pancreatic adenocarcinoma (PDAC) have a 5-year survival rate of 8%, the lowest of any cancer in the United States. Traditional chemotherapeutic regimens, such as gemcitabine- and fluorouracil-based regimens, often only prolong survival by months. Effective precision targeted therapy is therefore urgently needed to substantially improve survival. We utilized a platform to develop a novel combination of repurposed FDA approved drugs that would target pancreaticoduodenal homeobox1 (PDX1) and baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) utilizing super-promoters of these target genes to interrogate an FDA approved drug library. We identified and selected metformin, simvastatin and digoxin (C3) as a novel combination of FDA approved drugs, which were shown to effectively target PDX1 and BIRC5 in human PDAC tumors in mice with no toxicity.

Keywords

Weighted Gene Co-Expression Analysis; RNA Sequencing; BIRC5; PDX1, Super Promoter; Gaussia Luciferase; High Throughput Screening; FDA Approved Drug Libraries

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Introduction

Pancreatic cancer has a 5-year survival rate of 8%, the lowest of any cancer in the United States[1]. As early stages of the disease are often clinically and biochemically silent, patients typically present with advanced or metastatic disease at the time of diagnosis[2]. This late presentation, and lack of clinically successful drug therapies, maintain pancreatic ductal adenocarcinoma (PDAC) mortality rates that are similar to incidence rates.

Traditional chemotherapeutic regimens, such as gemcitabine- and fluorouracil-based regimens, are highly toxic and, even in the most optimal scenarios, often prolong survival by only weeks or months. Effective precision therapy is therefore urgently needed to substantially improve survival. Toward this end, there has been a recent paradigm shift in the treatment of solid tumors from traditional chemotherapies to more individualized molecular-targeted therapies. Cancer genomic profiling hold the potential to allow targeted therapies that will lead to the safest and most effective neoadjuvant or adjuvant treatment strategies for each individual patient[3].

Our laboratory was the first to identify that pancreaticoduodenal homeobox1 (PDX1) is an oncogenic transcription factor and potential target for PDAC. We developed PDX1 targeted gene therapy using an insulin super-promoter (SHIP) to drive viral thymidine kinase, as well as PDX1 RNAi therapy, both of which effectively ablate human PDAC tumors in mice [4-6]. The SHIP is activated by PDX1, therefore can be used as an assay for targeting PDX1[4-6]. Another well-studied cancer target gene is baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5; survivin). We generated a BIRC5 super-promoter (BIRC5-SP) to drive theranostic genes and demonstrated that this vector, coupled with an engineered viral delivery system, imaged and detected minute human PDAC tumors in mice for early detection of PDAC) [5, 7].

We hypothesized that target gene super-promoters could be used for high throughput screening (HTS) of an FDA approved drug library to identify drugs that would inhibit the target gene promoters. We engineered SHIP-luciferase and BIRC5-SP-luciferase vectors and cloned them into a PDAC cell line for HTS [5, 7]. When placed upstream of the luciferase reporter gene, both SHIP and BIRC5-SP markedly increased expression of luciferase reporter proteins, while still maintaining critical aspects of their normal cis-regulatory functionality. In this way, subtle regulatory changes in SHIP and BIRC5-SP activity caused by candidate drugs were detected. Thus a more sensitive HTS capable of detecting small changes in SHIP and BIRC5-SP regulation was achieved. Candidate drugs were tested alone and in combination for inhibition of PDAC proliferation and suppression of PDX1 and BIRC5 *in vitro*, followed by therapeutic responses *in vivo* in mouse models. For this study, we chose to combine simvastatin, digoxin and metformin for reasons listed in Table 1 and demonstrated that these three drugs synergistically suppress human PDAC cell proliferation *in vitro* and xenograft tumor volume *in vivo* via targeting of PDX1 and BIRC5.

Materials and Methods

Cell Lines, Vectors, and Antibodies

Cell lines: Human pancreatic cancer cell lines PANC-1, Mia PaCa2, and Capan2 were purchased from (American Type Culture Collection [ATCC], Bethesda, MD). Human embryonic kidney cells HEK 293FT (Thermo Fisher Scientific, MA), human primary pancreatic cells epithelial cells (HPPE) (Cell Biologics, Inc. Chicago, IL) were obtained. A human pancreatic ductal epithelial (HPDE) cell line was kindly provided by Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada) and authenticated by short tandem repeat (STR) analysis using Promega kit with 16 STR loci every 6 months. PDAC patient-derived cell lines (PDCL) were kindly provided by Andrew Biankin at Wolfson Wohl Cancer Research Centre & Institute of Cancer Sciences, Canada. PDCL5 (original TKCC-05) cells and PDCL15 (original TKCC-05-Lo) cells was maintained in conditional media, respectively (Supplemental method). The vectors and antibodies were described at Supplemental method.

Stable Transfection

Lentiviral CMV-Luc2tdT, $BIRC5^{EP}$- Luc2tdT, $BIRC5^{SP}$-Luc2tdT, EHIP- Luc2tdT, SHIP-Luc2tdT,were used to infect PDAC cells to generate stable cell lines. Briefly, culture media was replaced with fresh media supplemented with 8μg/ml polybrene (Sigma-Aldrich, St Louis, MO), and lentiviruses were added directly into the media. The media was replaced after 3 days and puromycin selection began at a concentration of 1μg/ml for 5 days.

High Throughput Screening (HTS)

HTS of United States Food and Drug Administration (FDA)-approved drugs was performed via the Molecular Screening Shared Resource at the University of California Los Angeles (UCLA). This platform queries the Prestwick Chemical Library® (http://www.prestwickchemical.fr/) of 1200 small molecules representing drugs approved for human use by the FDA, European Medicines Agency (EMA), and other regulatory agencies. The screen was performed in 384-well cell culture microplates designed to avoid microplate edge effects. 20 μl culture medium per well were dispensed into 384-well microtiter plates (Greiner One) and 0.5 μl of 1 mM test compound solution in dimethyl sulfoxide (DMSO) was added using a 500-nl V&P custom pin tool (San Diego, CA). In negative-control wells, 0.5 μl DMSO alone was added. 2000 Mia $PaCa2^{BIRC5\text{-}SP\text{-}Luc2tdT}$ vs Mia $PaCa2^{CMV\text{-}Luc2tdT}$, Mia $PaCa2^{SHIP\text{-}Luc2tdT}$ vs Mia $PaCa2^{CMV\text{-}Luc2tdT}$ cells were seeded in 384-well plate in 20μl culture medium per well (Phenol red-free DMEM supplemented with 4.5 g/l glucose, charcoal-treated fetal calf serum [FCS] 10%) in the presence of small molecule compounds (10 μM final concentration, DMSO concentration of 0.5% v/v). Cells were then incubated at 37°C, 5% CO_2. Twelve hours after cell seeding, the screens were performed in a TECAN robotic station (for cell handling, treatment and staining) and transferred via a Caliper Twister II robotic arm to coupling microplate stacks and imaged with the INCell1000 analyzer microscope (GE LifeSciences, Pittsburgh, PA).

Drug Combination Analyses

Drug analyses of synergism, additive effect or antagonism were performed based on PDAC cell viability assays. Cells were treated for 48h with metformin, simvastatin and/or digoxin, in single, double and triple combinations at increasing concentrations. Subsequently, using a mass-action law-based method that allows for automated computerized simulation of synergism and antagonism by the software Compusyn (version 1.0, ComboSyn Inc), the combinatorial effect of metformin, simvastatin and/or digoxin was determined by the calculation of Combination Index (CI). According to the CI theorem, values of CI < 1, CI = 1, and CI > 1 represent synergism, an additive effect and antagonism, respectively[8].

IncuCyte® Apoptosis Assay

IncuCyte® Caspase- 3/7 Green Apoptosis Reagent was purchased from Essen Bioscience). IncuCyte® Apoptosis Assay was performed following manufacture's instruction. Briefly, the PANC1 and HPDE cells were placed in the 96-well plate and incubated in 37°C for 24h. The cells were treated with C3 (Metformin 500μM, Simvastatin 4μM and Digoxin 50nM) vs PBS control followed by adding IncuCyte® Caspase- 3/7 Green Apoptosis Reagent at final concentration 5μM. The plate was placed in IncuCye and the scanning was started every 2h. The data were analyzed and presented by IncuCyte® Analysis Software.

Optical Imaging and Serum GLuc measurement

Animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Ten to 8-week-old male nude mice (Charles River Laboratories, Inc. Wilmington, MA) were subcutaneously implanted with $1x10^6$ Mia PaCa2 or Capan2 cells stably expressing GLuc-2A-sr39TK with Matrigel. Optical imaging was performed with an IVIS CCD camera (IVIS Lumina II, Perkin Elmer, Waltham, MA) on day 8, 14, 21, and 30. Mice were anesthetized with isoflurane, and then provided 100 μg/50 μl of water-soluble coelenterazine per mouse (Nanolight Technology, Pinetop, AZ) via tail vein injection and immediately imaged and analyzed using the Living Image Software (Caliper Life Sciences). For measurement of serum GLuc levels, 50 μl blood per mouse were collected from xenograft tumor mice via tail vein on days 2, 4, 7, 14, 21, and 30 after cancer cell implantation and serum was extracted; 50 μl serum was used for evaluation of GLuc levels.

WGCNA Analysis.

Weighted Gene Co-expression Network Analysis (WGCNA) was used for scale-free network topology analysis of RNA-seq data[9]. WGCNA R package was used to cluster highly correlated genes and to determine the clusters whose gene expression was correlated with the traits examined. An adjacency matrix based on expression correlation was created using a soft threshold procedure to allow a scale free topology. The clusters created by WGCNA were defined as modules, and the minimum number of genes in a module was set to 30. Throughout the analysis, the functional annotation tool DAVID Bioinformatics Resources 6.7 was used to determine gene ontology terms enriched by a list of genes. DAVID analyses were performed on the lists of genes corresponding to significant WGCNA modules.

Statistical Analysis

Respective experimental group comparisons were evaluated by one-way ANOVA using GraphPad Prism. All other data, including those collected during western blot, immunohistochemistry, and reporter assay experiments were analyzed by two-tailed t-test using Microsoft Excel. P values < 0.05 were deemed significant. Unless otherwise specified, error bars indicate standard deviation.

RESULTS

Selection of PDX1 and BIRC5 as PDAC targets for HTS of FDA approved drugs using SHIP-FLuc and BIRC5-SP-FLuc assays

We performed weighted gene co-expression network analysis (WGCNA) on 11 PDAC specimens as well as patient-matched benign pancreas specimens to identify PDX1 and BIRC5 as targets for PDAC therapy[10]. First, we identified BIRC5 as a target for PDAC therapy through RNA-Seq analysis of 11 PDAC specimens as well as patient-matched benign pancreas specimens. These data were subjected to weighted gene co-expression analysis to identify a network of hub genes that serve as potential PDAC biomarkers or targets (Figure 1A). High expression of PDX1 (Figure 1B) and BIRC5 (Figure 1C) was further validated by IHC staining of human pancreatic specimens. On average, 75.7% and 89.3% of patient cells derived from the PDAC specimens showed strong positive signals for PDX1 and BIRC5, respectively, suggesting both PDX1 and BIRC5 may serve as target genes (Figure 1B and 1C, bar graph).

Next, we designed SHIP-Fluc and BIRC5-SP-FLuc, as previously described [5, 7]. Previous work demonstrates use of SHIP and BIRC5-SP results in promoter activity that is more than 100-fold higher than endogenous insulin and BIRC5 promoter activities in PDAC cells, rendering these super promoters capable of detecting subtle changes in SHIP and BIRC5 activity caused by exposure to small molecules [7]. In order to define the optimal time point for evaluation of BIRC5-SP activity, we performed promoter reporter assay timelines in IncuCyte. BIRC5-SP-EGFP vs CMV-EGFP stably transfected Mia PaCa2 cells were plated on 24 well plates, which were immediately cultured in IncuCyte for 48h. As shown in Figure 2A, starting at 15 h after cell culturing, the BIRC5 activity demonstrated higher activity than the CMV promoter ($p<0.05$), indicating that from this time point we would observe the changes of BIRC5 promoter's activity level following application of BIRC5 inhibitors (Fig 2A). Next, we treated Mia PaCa2 cells which carried BIRC5-SP-GLuc or CMV-GLuc using YM155, a known BIRC5 promoter inhibitor [11, 12]. We used a minimal dose (1nM) of YM155 to demonstrate whether YM155 suppressed promoter activity without affecting cell viability. 30 ul/per well of cell culture medium were taken for GLuc measurement. Remaining cells was used to evaluate cell viability by CellTiter Glo assay. Results showed that BIRC5-SP-GLuc levels had significantly decreased levels of GLuc on 24h and 48h after treatment as compared to the CMV-GLuc; BIRC5-SP-GLuc had 5.4, 2.19 and 0.97 fold decreased GLuc levels than CMV-GLuc at 24h, 48h, and 72h, respectively. The lowest BIRC5-SP-GLuc level was 56.3 % at 48h after treatment, which was consistent with the lowest cell viability at 77.3% (Figure 2B). Therefore, we chose the time point of 24h after treatment as an optimal timepoint to evaluate BIRC5-SP activity in the HTS assay.

For HTS, stably transfected SHIP-FLuc and BIRC5-SP-FLuc Mia PaCa2 cell lines were used to survey the Prestwick drug library of 2500 FDA approved drugs. Chemicals were applied to cells plated on 384-well plates (2000 cells/well). We specifically screened the library for SHIP- and BIRC5-SP-inhibitory drugs using FLuc reporter assays. A 4 standard deviation cutoff was utilized for hit identification and plate internal normalization. In this way, we identified a panel of small molecule drugs that significantly inhibit SHIP-FLuc and BIRC5-SP-FLuc activity (Figure 2C) and groups of drugs were listed in Figure 2D based on inhibing more than 70% of SHIP-FLuc and BIRC5-SP-FLuc activity. We found that simvastatin and digoxin inhibited both SHIP and BIRC5-SP activity. Metformin inhibited SHIP-Fluc, but not BIRC5-SP-Fluc. However, metformin is a well characterized small molecule for cancer therapy that has been previously studied in our research group and shown significantly suppress of PDAC cell lines via PDX1 [13-17]. Based on the typical non-cancer treatment doses and favorable safety and toxicity profiles, three non-chemotherapeutic drugs metformin, simvastatin and digoxin (C3) were selected for further study based on the criteria in Table 1 [15, 16, 18-22]. Paclitaxel was identified within this cohort was selected to also study the potential of C3 as an adjuvant to paclitaxel chemotherapy.

Validation of metformin, simvastatin, digoxin inhibitory effect on PDAC cells

The individual inhibitory effects of metformin, simvastatin and digoxin on PDAC cells were first investigated via dose-response experiments. Simvastatin and digoxin were compared to other statin and cardiac glycoside analogs. Dose-response curves demonstrated that simvastatin had similar cytotoxic effects on PANC1 cells compared to its analogs, whereas digoxin demonstrated a more efficacious inhibitory effect than ouabain (Figure 3A). Furthermore, the 50% inhibitory concentration (IC50) doses were determined in PANC1, Mia PaCa2, PDCL5 and PDCL15 showing variation of the drug responses in different PDAC cell lines (Figure 3B). PANC1 (Figure 3C) and Mia PaCa2 (Figure 3D) cells had varied responses to chemotherapeutic drugs. Both cells had low sensitivity to paclitaxel (Figure 3C and D). The variations suggest that one drug alone would not efficiently inhibit growth of all PDAC cell lines. As demonstrated in Figure 3E, drug combinations with 2 drugs or 3 drugs largely contribute to cytotoxicity of PDAC cells through addition, synergy, or complementary effect. Metformin, simvastatin and digoxin (C3) had a synergistic effect and was used in subsequent *in vitro* and *in vivo* studies. Overall, paclitaxel alone (100 nM) had a low cytotoxic effect on PDAC cells, however, when combined with C3, all PDAC cells, except PDCL5, had significantly increase cell death as compared to C3 alone ($p<0.05$) (Figure 3E). To validate BIRC5-SP's advantage over the endogenous promoter (EP) in screening FDA approved drugs, we performed the GLuc reporter assay compared to both BIRC5-EP and BIRC5-SP activity after C3 treatment in PANC1 cells. BIRC5-SP had more than 15-fold higher activity than BIRC5-EP ($p<0.05$). Compared to controls, C3 resulted in more than 8.2-fold decrease of BIRC5-SP activity, as compared to 2.7-fold decrease of BIRC5-EP activity by C3 ($p<0.05$); Figure 3F). The data demonstrate that BIRC5-SP is more sensitive to the inhibitory drugs than BIRC5-EP, thus suggesting that the use of a target super promoter is important in identifying FDA approved drugs during screening.

Demonstration of C3 synergistic effect on PDAC cells

We next analyzed the effects of C3 on PDAC cell growth by determining the interactions between single, two and three drug combinations in constant ratios. The doses were chosen based on IC50 of each drug, which was determined in PDAC cells as shown in Figure 3B. For *in vitro* cancer cell treatment experiments, dosages of metformin used ranged from 0.3 ~ 40 mM on various cancer cell lines [23-25]. We chose 500 μM for PDAC cell lines based on IC50 tests on pancreatic cancer cells. Dosages of simvastatin ranged from 250 nM ~ 32μM on various cancer types[26, 27]. We chose 4 μM for PDAC cell lines based on IC50 tests on PDAC cells. The dose range of digoxin was 10 ~ 500 nM in cancer cell lines in other studies. We chose 50nM for the *in vitro* PDAC cell line studies based on IC50 tests on PDAC cells[28]. The chosen drug concentrations and combinations used in the synergistic effect analysis are shown in Figure 4A. Cell viability was evaluated using CellTiter Glo and the CI was calculated using the method of Chou-Talalay[8]. At the 1×level (i.e. IC50) all combinations produced synergistic effects (CI<1) (Figure 4B) (Figure S1, S2). Interestingly, higher drug concentrations did not always produce greater synergistic effects. For example, 8x drug concentrations (i.e. 8 × IC50) in PDCL5 and Mia PaCa2 PDAC cell lines did not show increased synergy. Similarly, 2× drug concentrations in the PANC-1 PDAC cell line did not show increased synergy between the three drugs. This is likely due to the cytotoxic effect of a single drug overshadowing any potential synergy at such high doses. We also noted that the CI value varied among drug combinations between different PDAC cells (Figure 4C). At the 1× level, it was noted that three variations of the two-drug combination in the same cell lines or the two-drug combination at different cell lines had varied synergistic effect, indicating heterogeneity of PDAC cells in responding to drug combinations (Figure 4D). These data demonstrate that C3 had a greater synergistic effect overall that results in cell death when compared to any individual or two drug combination.

Demonstration of C3 suppressing PDAC tumor growth by targeting PDX1 and BIRC5

In order to evaluate C3's therapeutic effect on PDAC cells *in vivo*, Human PDAC xenografts were generated by subcutaneous injection of $1x10^6$ cells of either Mia PaCa2 or PDCL-15 cells. BIRC5-SP-GLuc stably transfected PDCL-15 (PDCL-$15^{BIR5\text{-}SP\text{-}Gluc}$) or Mia PaCa2 (Mia PaCa$215^{BIR5\text{-}SP\text{-}Gluc}$) cells into nude/nude mice. 10 mice (5 males; 5 females) per group were used, which provided 80% power to measure tumor volume and detect serological GLuc and imaging reporter. Our previous study demonstrated that rising GLuc levels highly correlate with increasing PDCL tumor volume. Therefore serologic GLuc can be used to track tumor volume and response to therapy in real time *in vivo* [7]. Two weeks after cell inoculation, treatment with C3 was performed by intraperitoneal injection of metformin (100 mg/kg), simvastatin (20 mg/kg), and digoxin (2 mg/kg) in PBS (200 μl/mouse) daily for 2 weeks. PBS served as control. The *in vivo* dosages of metformin, simvastatin, and digoxin were determined from IC50 tests and published studies by other groups (Table 2). For the *in vivo* treatment experiments, metformin was delivered intraperitoneally at a dose of 100 mg/kg body weight, since similar dosages of 50~250 mg/kg have been used in another animal test and the lethal dose for mouse (LD50) is 477 mg/kg [16, 29]. The equivalent dose for metformin in clinical trials is 500 mg/day for a person at 60 kg body weight, which is in the range of doses used for daily oral therapy 500 ~ 2000 mg/day. Simvastatin was administrated via intraperitoneal injection at 20 mg/kg body

weight [21], whereas the lethal dose for mouse (LD50) of simvastatin is 798 mg/kg (Table 2). The equivalent dose of simvastatin for patients is 100 mg/day for a person (60 kg body weight), which is higher than oral doses used for simvastatin in patients (5 ~ 40 mg/day). Digoxin was delivered intraperitoneally at a dose of 2 mg/kg body weight, since the same dosage was used in other mouse studies, whereas the lethal mouse dose (LD50) of digoxin is 4 mg/kg [30]. The equivalent clinical dose of digoxin at 2 mg/kg body weight (60 kg body weight) is also higher than the oral dose used clinically (750 ~ 1250 mcg/day).

As shown in Figure 5A, PDAC tumor growth was inhibited in the C3 treatment group with no growth over 28 days (Figure 5A; blue line) as compared to the PBS control group (Figure 5A; red line). Correspondingly, the serum GLuc levels significantly increased in the PBS control group over 28 days (Figure 5A; red bar). There was an insignificant increase of GLuc (Figure 5A; blue bar) in the C3 group. Tumor volume was also evaluated using bioluminescence imaging at 28d after C3 therapy showing significantly reduced signals in the C3 treatment group (Figure 5B). Tumor diameter correlated with serologic GLuc levels (5A; blue bar) and bioluminescence imaging (5C; right bar). Thus, the response to C3 in mice was monitored in real time using serologic GLuc levels, which correlated with bioluminescence imaging. The data demonstrate that C3 significantly suppressed PDCL-15 tumor growth compared to controls with no overt toxicity observed in any mice. In the Mia PaCa2 model, C3 resulted in a significant decrease of tumor volume on day 21 ($P<0.05$) and C3 combined with paclitaxel significantly enhanced tumor suppression as compared to C3 alone or paclitaxel alone ($p<0.05$) (C). These data suggest that C3 has potential for therapy for PDAC alone and as an adjunct with chemotherapy.

To determine whether C3 therapy inhibited BIRC5 expression, BIRC5 promoter activity was evaluated using BIRC5-SP-GFP stably transfected Mia PaCa2 cells that were treated with C3. GFP expression was subsequently monitored in IncuCyte for 48h. BIRC5 mRNA levels and protein levels were evaluated by real-time PCR, and protein levels were determined by western blot and immunofluorescence. C3 therapy resulted in significantly reduced PDX1 mRNA and BIRC5 mRNA expression in PDCL5 cells (Figure 5D) and PDX1 and BIRC5 protein levels in PANC1 cells (Figure 5E) after C3 treatment. Compared to single-drug treatment, C3 showed the greatest inhibition of BIRC5 expression. C3 inhibited BIRC5 expression in all PDAC cell types tested (Figures 5D and E).

To test C3 against BIRC5 expression in real time, Mia PaCa2 cells stably transfected with BIRC5-SP-GFP were treated with C3 and GFP expression was monitored every 2h using IncuCyte. C3 resulted in significant suppression of GFP expression in BIRC5-SP-GFP cells (Figure 5F; blue line), thus demonstrating that C3 effectively inhibits BIRC5-SP activity in Mia PaCa2 cells and that the response to C3 can be monitored in real time using a BIRC5-SP-GFP assay. Significantly reduced PDX1 and BIRC5 expression were also observed *in vivo* in PDCL15 tumors by immunofluorescent staining using anti-PDX1 and anti-BIRC5 antibodies (Figure 5F). These data support the hypothesis that C3 suppresses PDAC tumor growth via targeted inhibition of PDX1 and BIRC5 expression.

Mechanisms by which C3 treatment suppresses PDAC

To gain insight into mechanisms of C3 therapy against PDAC, several techniques were utilized, including IHC, RNA-seq and QPCR and weighted gene co-expression analysis (WGCNA). As seen in Figure 6, C3 therapy in xenograft mice nearly completely suppressed PDX1 and BIRC5 (survivin) protein expression in PDCL15 tumors in mice. Inhibition of PDX1 and BIRC5 expression by C3 treatment resulted in increased cell apoptosis, as seen in Figures 6A and B by measuring caspase 3/7 of PANC1 cells using IncuCyte and identification of apoptotic cells in PDCL15 xenograft tumor using TUNEL assay after C3 treatment, respectively. No significant apoptosis was observed in benign human pancreatic ductal (HPDE) cells after C3 treatment (Figure S3), suggesting that inhibition of PDX1 and BIRC5 leads to cell apoptosis in PDAC cells, but not in benign HPDE cells, which lack expression of PDX1 and BIRC5. C3 therapy also resulted in a significant decrease of Ki67 expression in PDCL15 xenograft tumor *in vivo* (Figure 6C), demonstrating that inhibition of PDX1 and BIRC5 affected cell proliferation. To explore signaling pathways affected by C3 therapy, RNA-Seq was performed in PDCL5 cell lines and PDCL15 xenograft tumors, followed by WGCNA. Based on the RNA-Seq analysis and WGCNA, C3 therapy affected three major networks, which included cell cycle, ATP energy and cell death networks (Figure S4-8, Figure 6D, E and F). Two genes from each network were chosen for validation by QPCR; C3 significantly decreased expression of BIRC5 and TOP2A in the cell cycle protein network (Fig. 6D), decreased expression of SEMA7A and DDX5 in the ATP energy network (Figure 6E) and increased DUSP15 and RHOB in the cell death network (Figure 6F). PDCL15 xenograft tumors treated with C3 therapy demonstrated decreased cell proliferation enriched genes (Figure S11-12, Figure 6G). Three of these genes were further validated using QPC; FLNB, TOP2A and BIRC5 were suppressed in PDCL15 tumors following C3 therapy. These studies demonstrate that C3 targets PDX1 and BIRC5 and involves cell cycle, ATP energy and cell death networks.

To study whether PDX1 activates BIRC5 promoter to regulate BIRC5 expression, we performed reporter assays by transient transfection of BIRC5-SP-GLuc and PDX1 expression vector in PANC1 and PDCL15 cells. 48h after transfection, the GLuc levels in cell media and cell proliferation were determined by bioluminescence assay and Celltiter Glo, respectively. The results showed that GLuc levels significantly increased in both cell lines after overexpression of PDX1 (Fig7A, $p<0.05$), suggesting that PDX1 activates the BIRC5 promoter. Increased cell proliferation in both PDAC cell lines was observed after over expression of PDX1 (Fig7B, $p<0.05$), which also suggests PDX1 up-regulates BIRC5 expression and is consistent with our previous results[6, 31, 32]. The data suggested that C3 therapy inhibits PDX1, which in turn reduces BIRC5 expression, thus leading to decreased cell proliferation, increased cell apoptosis and cell death via reduction of cell cycle and ATP-related gene expression and enhancement of cell death genes.

Discussion

In search of targeted, non-toxic therapy for PDAC using a novel combination of repurposed FDA approved drugs, we determined that metformin, simvastatin and digoxin (C3 therapy) effectively target PDX1 and BIRC5 in human PDAC tumors in mice. The platform used to

identify the C3 combination includes 1) selecting PDX1 and BIRC5 as PDAC targets, 2) utilizing super-promoters of the target genes to drive a reporter gene assay to perform HTS of an FDA approved drug library in order to identify potential drug candidates, 3) screening drug combinations against PDAC cell lines and selecting an optimal combination (C3) based upon several criteria, 4) testing C3 in patient-derived and commercial PDAC cell lines and xenograft mouse models, and 5) evaluating mechanisms of action. The pre-clinical studies demonstrate that C3 suppresses PDAC tumor growth in mice via near complete suppression of PDX1 and BIRC5 expression, as well as augments the effect of low dose paclitaxel on PDAC *in vitro* and *in vivo*. Therefore, C3 therapy holds potential as PDAC targeted therapy and to augment the effect of known PDAC chemotherapy.

We chose metformin, simvastatin and digoxin, since the dosages, safety and toxicity profiles are well known via clinical trials, clinical use and package inserts. Furthermore, these drugs are generic, inexpensive and all have been shown to have anti-cancer properties by inhibiting important enzymes in molecular cancer pathways (Table 1).

Metformin is a first-line medication for the treatment of type 2 diabetes, particularly in people who are overweight. It is also used in the treatment of polycystic ovary syndrome [33], prevent the cardiovascular disease and cancer complications from diabetes [34] [35]. Metformin acts on PDAC via multiple signaling pathways, including AMP-activated protein kinase, mammalian target of rapamycin, insulin-like growth factor, c-Jun N-terminal kinase/ mitogen-activated protein kinase (p38 MAPK), human epidermal growth factor receptor-2, and nuclear factor kappaB pathways [13]. Patients on metformin have a lower incidence of PDAC, thus metformin demonstrats promise of being an effective PDAC therapy. However randomized clinical trials have not shown a benefit of metformin as a single agent. Our studies demonstrate that metformin inhibits PDX1 and BIRC5 promoter activity that results in an increase of cell death, which is probably due to upstream signal regulations such as nuclear factor kappaB or MAPK pathways.

Statins reduce serum cholesterol levels and mevalonate synthesis by inhibiting HMG-CoA reductase, which is involved in the isoprenylation of the intracellular G-proteins Ras and Rho to regulate cell proliferation, differentiation, and apoptosis. Simvastatin is one of the most highly used drugs in the US, and the safety profile has been firmly established. The efficacy of statins as anticancer agents has been evaluated in both monotherapy and combination therapy with currently used chemotherapeutic drugs. These studies have shown the potential mortality benefits of statin consumption in patients with different types of cancers, including esophageal, breast, lung, liver, pancreatic, endometrial, and colorectal cancer [36]. Statins induce tumor-specific apoptosis through mitochondrial apoptotic signaling pathways, which are activated by the suppression of mevalonate or geranylgeranyl pyrophosphate (GGPP) biosynthesis[36]. Recent studies showed that targeting the mevalonate pathway by statins overcame acquired anti-HER2 treatment resistance in breast cancer through suppression of downstream survivin expression[37]. This is consistent with our findings that simvastatin induced cell apoptosis by inhibition of BIRC5 expression.

Digoxin is a cardiac glycoside, which modulates the Na^+/K^+-ATPase protein complex upon binding and activate associated downstream signaling pathways, such as phospholipase C, mitogen-activated protein kinase (MAPK), phosphatidyl-inositol-3-kinase and Src kinase signaling cascades. A recent study showed that cardiac glycoside drugs reduce p53 levels by initiating Src/MAPK signaling pathways and inhibiting p53 protein synthesis [19]. Digoxin is a long-standing effective cardiac support medication, known also as digitalis, which was used extensively for mild to moderate heart failure. Digoxin is considered by WHO an 'Essential medicine" based on its safety, efficacy and pharmacoeconomic benefit. Digoxin suppresses PDAC via inhibition of Na^+/K^+-ATPase and other pathways in multiple pre-clinical studies, but, has not been tested in clinical trials for PDAC.

Cell based assay using tissue or cancer specific promoter driving reporter genes for HTS has been employed for years[38-40]. Endogenous target gene promoters are routinely used, however high noise-background signaling frequently masks the inhibitory effect of the drug(s) on the target gene promoter. Our data suggest that the use of a target gene super-promoter is superior to BIRC5 endogenous promoter, thus permitting a marked sensitivity for identifying FDA approved drugs to be repurposed [4, 41-43]. We utilized super-promoters for the two selected targets PDX1 and BIRC5 in order to identify FDA drugs that inhibit these promoters.

PDX1 is a homeodomain-containing transcription factor and is well known to be a master regulator for expression of genes crucial for both exocrine and endocrine pancreatic development, β-cell maturation, and maintenance of normal β-cell function[44-48]. There is significant evidence supporting the hypothesis that *PDX1* is an oncogenic transcription factor, as defined by Darnell, regulating PDAC [32, 42, 49-52]. We have shown PDX1 is overexpressed in more than 250 human PC specimens, which is consistent with other studies showing that 50-100% of PC overexpress PDX1 and is associated with advanced clinical pathological stages and poor prognosis[6, 31, 53, 54] [55]. PDX1 regulates proliferation and invasion of human PDAC cell lines and mouse models [32, 42, 49-52]. Microarray analysis revealed that PDX1 overexpression disrupted cell cycle proteins including CyclinE, CDK2, p21 and p27, both *in vitro* and *in vivo*, suggesting an important oncogenic mechanism of *PDX1*. Our published data from PDAC cell lines, preclinical animal models and PDAC specimens support the hypothesis that PDX1 is an oncogenic transcription factor regulating PDAC, therefore is a target for PDAC [6, 32, 42, 52, 56].

BIRC5 is the smallest member of the inhibitor of apoptosis (IAP) family of proteins, involved in inhibition of apoptosis and regulation of cell cycle. BIRC5 is prominently expressed during embryonal development, absent in most normal, terminally differentiated tissues but upregulated in a variety of human cancers [57-62]. BIRC5 is over-expressed in 77-94% of PDAC but absent in benign pancreatic tissue [63-67]. Expression of BIRC5 in cancers correlates with not only inhibition of apoptosis [68, 69] and decreased rate of cell death [70-73], but also resistance to chemotherapy and aggressiveness of tumors [74, 75]. Regulatory mechanisms of BIRC5 expression are not fully understood. At the transcriptional level, BIRC5 expression has been demonstrated to involve cell-cycle-dependent element/cell cycle gene homology region (CDE/CHR), p53/E2F binding sites[76]. Transcription factors NF-kB, STAT3 (Signal Transducer and Activator of Transcription) and KLF5 (Kruppel-

like factor 5) and Hypoxia-inducible Factor-1α (HIF-1α) contribute to direct activation of survivin [77, 78]. In addition, signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt pathway, mitogen-activated protein kinase (MAPK) pathway are participating in the regulation of BIRC5 [79]. Our study demonstrated that PDX1 overexpression upregulated BIRC5 promoter activity. Whether PDX1 has a direct regulatory action to BIRC5 promoter remained to be determined, although several presumed PDX1 binding sites have been found on the BIRC5 promoter.

Strategies engaged in targeting BIRC5 have been proposed and studied: 1) immunotherapeutic approaches to induce immune response against BIRC5, 2) small molecule inhibitors/antagonists to suppress BIRC5 expression and block function of BIRC5, 3) nucleic acid based approaches to interfere with BIRC5 gene expression, or 4) gene ablation of BIRC5 to regulate cell cycle and apoptosis. Several small molecule inhibitors targeting BIRC5 have also been evaluated in various studies. YM-155 is a novel, small molecule that suppresses transactivation of BIRC5 through direct binding to its promoter, resulting in selective suppression of BIRC5 expression and induction of apoptosis in p53-deficient cancer cells *in vitro* [12]. YM155 has also shown to be effective in *in vivo* models of prostate, pancreatic, and lung cancer [11, 80, 81] and is the only BIRC5 inhibitor that entered Phase II clinical trials in 2015. However, it's trials were stopped due to off-target toxicity[82]. Other BIRC5 inhibitors such as terameprocol (EM-1421)[83], GDP366[84], and FL118 [85] have been tested in various cancer *in vitro* and *in vivo* studies showing the effective suppression of BIRC5 expression and inducing cell apoptosis, however none of the targeted therapies are being used clinically.

Our data demonstrate the combination of three drugs, metformin, simvastatin and digoxin, (C3), resulted in synergistic suppression of PDAC cell proliferation *in vitro* and inhibition of patient-derived PDAC tumor growth in mice with no overt toxicity seen in any of the mice. We demonstrated that C3 treatment inhibited the BIRC5 promoter, with suppression of BIRC5 RNA and PDX1 RNA and near complete suppression of BIRC5 (survivin) protein and PDX1 protein in patient-derived PDAC tumors in mice. Our data reveal that while C3 has an inhibitory effect on the growth of PDAC cells and tumors, the individual inhibitory effects and IC50 dose response of metformin, simvastatin and digoxin varied in each PDCLs vs commercial PDAC cell lines, suggesting driver mutation profiles and downstream pathways influencing the C3 effect. Our *in vivo* data demonstrate that C3 suppressed PDAC tumor growth via targeted inhibition of PDX1 and BIRC5 expression. In addition, the response to C3 *in vivo* was monitored in real time using secreted BIRC5 serum levels from PDAC cells engineered with BIRC5-SP-GLuc-2A-tdTomato assay via Incucyte, which demonstrates a novel non-invasive assay to measure *in vivo* tumor responses to therapy.

Our data demonstrate the feasibility of the platform to identify a combination of BIRC5-inhibitory FDA approved drugs using PDX1 and BIRC5 super-promoter high throughput screening assays. This method of identification of C3 holds the potential to identify precision cancer therapy at a low cost and in a clinically relevant timeframe. Although targeted drug development has improved clinical outcomes for a variety of cancer-related diseases, PDAC has yet to realize clinically relevant improvements using modern drug

design platforms. Novel molecularly-targeted drug therapies for PDAC frequently undergo costly and time-consuming developmental processes, only to ultimately fail to demonstrate efficacy or safety in clinical trials. New drug approval rates have been in decline since the 1990s with oncology drugs having the lowest likelihood of approval (i.e., 6.7%) from Phase 1 clinical trials [86]. Cumulatively, this creates a uniquely disadvantageous landscape for PDAC drug development, further complicating intervention efforts for a disease that is notoriously difficult to treat. Delays and barriers mean that translation of a promising molecule into an approved drug often takes more than 14 years and millions of dollars with low probability of success. Therefore, there is an urgent need to advance strategies to reduce the time frame and costs and improve success rates of targeted therapy, especially for lethal diseases such as PDAC.

Identification of FDA approved drugs in a clinically relevant timeframe that can be repurposed for targeted and non-toxic PDAC therapy is an exciting possibility. We propose that this HTS method could expedite PDAC therapy development by identifying current C3 FDA-approved medications capable of targeting PDX1 and BIRC5 promoters, since the dosing, safety and toxicity of these FDA approved drugs is well known. By coupling this strategy with patient-specific proteo-genomic sequencing data, we hope to advance precision therapeutics for PDAC patients in a clinically relevant timeframe.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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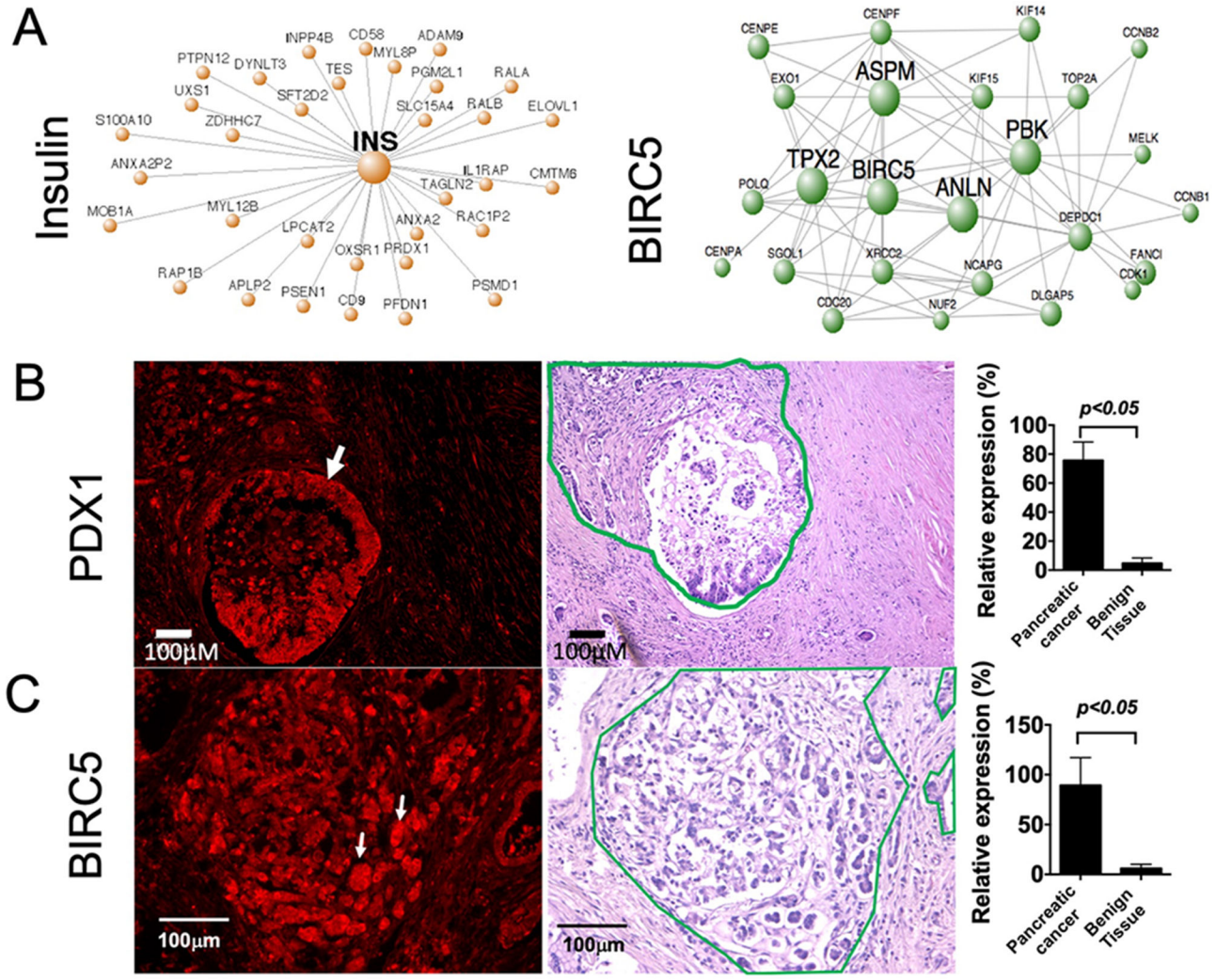


Fig. 1. (A) Identifying targets for PDAC therapy.

Weighted gene co-expression network analysis (WGCNA) of RNAseq data, from 11 PDAC specimens as well as patient-matched benign pancreas specimens showed that BIRC5 was a major hub gene in the cell cycle related network. IF staining of PDAC specimens using PDX1 and BIRC5 monoclonal antibody revealed overexpression of PDX1 (B) and BIRC5 (C) in human PDAC (white arrows on the IF image within highlighted area of matched HE staining image) vs a few positive for PDX1 and BIRC5 in adjacent benign tissues (outside circled area), with significant difference ($P < 0.05$) (Bar graph on B and C).

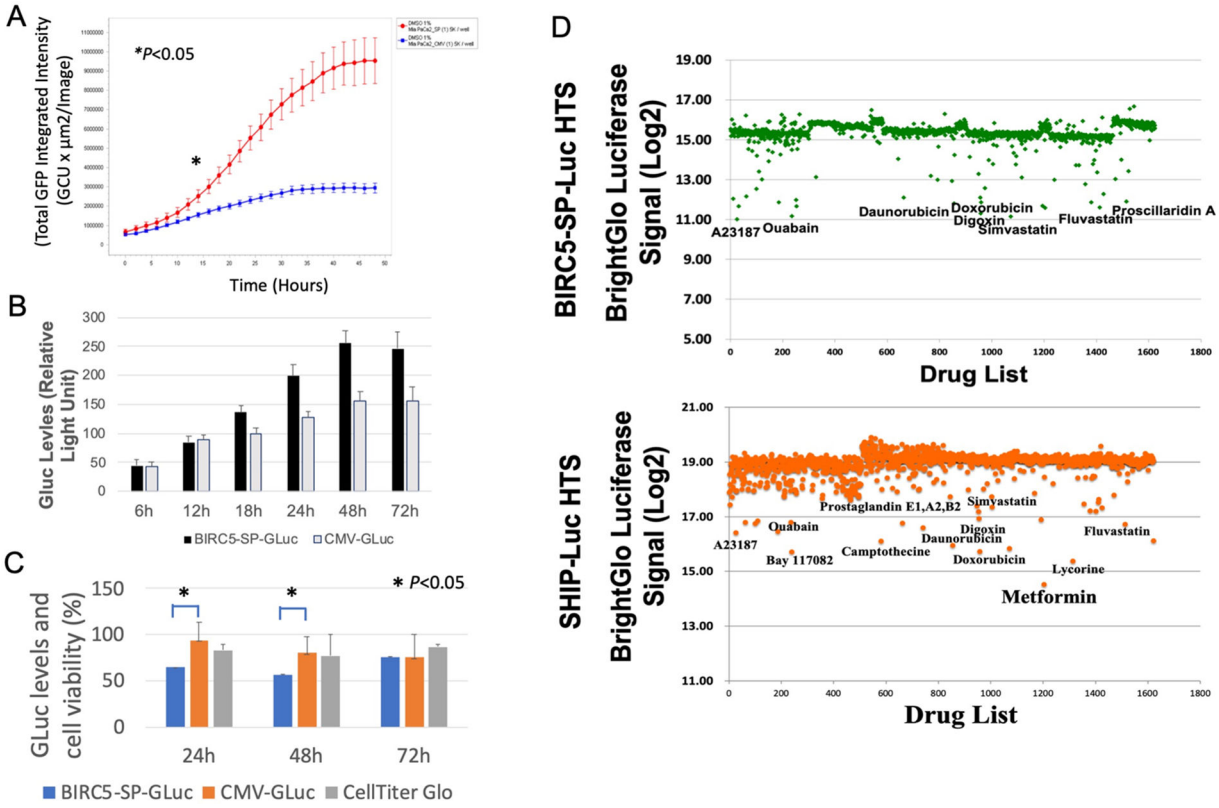


Fig. 2. Determining non-chemotherapeutic drugs targeting PDX1 and BIRC5 using HTS of FDA approved drug library.

IncuCyte monitors GFP expression in Mia PaCa2 cells carrying BIRC5-SP-GFP vs CMV-GFP showing that starting at 15 h after cell culturing, BIRC5 activity had significantly higher activity than the CMV promoter ($p < 0.05$) (A); PANC1 cells carrying BIRC5-SP-GLuc vs CMV-GLuc revealing that BIRC5-SP promoter activity had much greater than CMV promoter on 18 h, 24 h, and 48 h after culturing ($p < 0.05$) (B); BIRC5 inhibitor, YM155, significantly inhibited BIRC5 promoter activity at 24 h after treatment, but did not affect cell viability (C). HTS of FDA approved drugs using SHIP-FLuc and BIRC5-SP-FLuc assay was plotted(D).

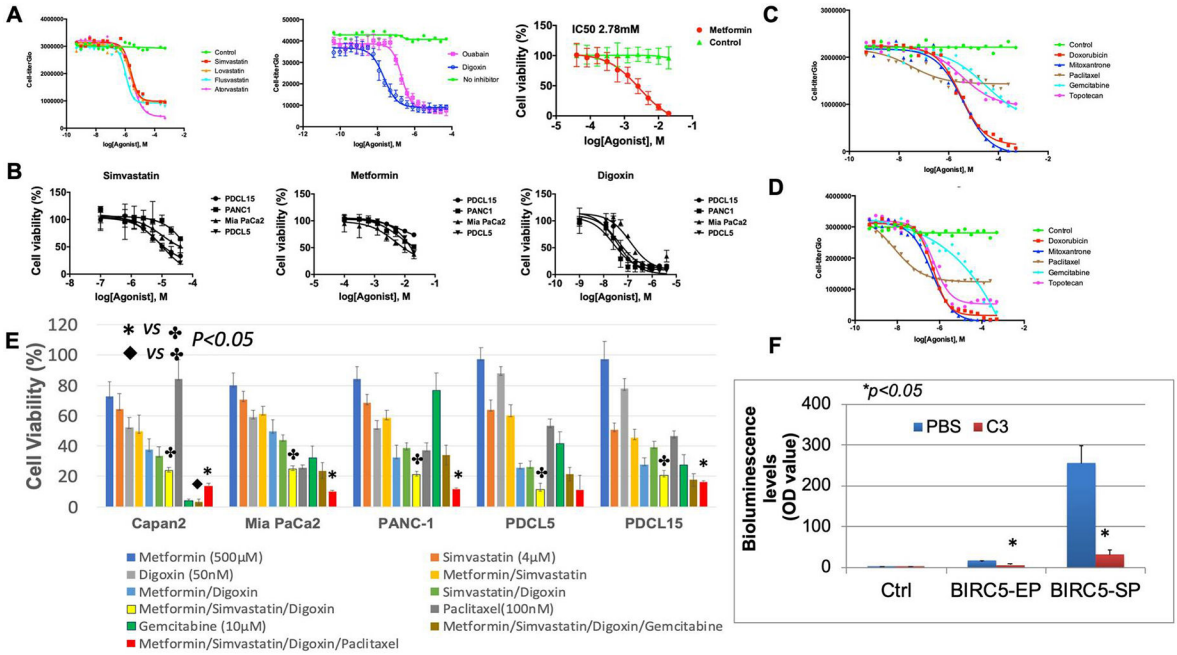


Fig. 3. Validation of cytotoxicity of single or combinations of simvastatin, digoxin, metformin and paclitaxel to PDAC cells.

Simvastatin and digoxin were compared to their respective chemical statin and cardiac glycoside analogs (A). Dose courses of simvastatin, metformin and digoxin were performed on PANC1, Mia PaCa2, PDCL5 and PDCL15 cell lines (B). Dose courses of chemotherapeutic drugs were carried out in PANC1 (C) and Mia PaCa2 (D) cells. Drug combinations of 3 non-chemo-drugs (C3) largely contributed to cytotoxicity of PDAC cells through additive, synergistic or complementary effects (D). C3 combined with paclitaxel, not gemcitabine, resulted in significant cell death as compared to C3 alone in all cells except PDCL5 ($p < 0.05$) (E). GLuc reporter assay showed that BIRC5-SP was more sensitive to respond to C3 treatment as compared to BIRC5-EP ($p < 0.05$) (F).

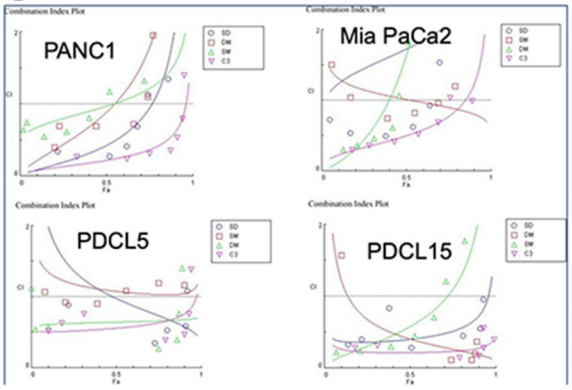
A

Drug combinations in constant ratio

- Sim: Simvastatin
- Dig: Digoxin
- Met: Metformin
- SD: Simvastatin+Digoxin
- DM: Digoxin+Metformin
- SM: Simvastatin+Metformin
- C3: Simvastatin+Digoxin+Metformin

	Sim (uM)	Dig (nM)	Met (mM)
8x	40.00	400.00	20.00
4x	20.00	200.00	10.00
2x	10.00	100.00	5.00
1x	5.00	50.00	2.50
1/2	2.50	25.00	1.25
1/4	1.25	12.50	0.63
1/8	0.63	6.25	0.31

B



C

Total Dose	PANC1 CI Value	Mia PaC2 CI Value	PDCL5 CI Value	PDCL15 CI Value
20040.4	2.07973	1.05788	1.50404	1.11590
10020.2	1.26733	0.76335	0.83441	0.73365
5010.1	1.10212	0.66321	0.53446	0.44939
2505.05	0.68024	0.61562	0.50807	0.35029
1252.53	0.39908	0.65152	0.99931	0.47005
626.263	0.21866	0.54387	0.83224	0.24266
313.131	0.24485	0.31678	0.68302	0.14884

D

Drugs combination index (CI) and effect at 1x (Sim 5uM+Dig 50nM+Met 2.5mM) drugs combination (CI<1 represents synergism)

	SD		DM		SM		C3	
	CI	Cell death	CI	Cell death	CI	Cell death	CI	Cell death
PDCL15	0.82631	38%	0.11392	74%	0.44604	53%	0.35029	90%
PDCL5	0.34254	73%	0.27046	75%	0.90007	39%	0.50807	79%
PANC1	0.68399	68%	0.72163	66%	0.60995	27%	0.68024	87%
Mia PaCa2	0.32744	38%	0.66929	42%	0.42978	39%	0.61562	57%

Fig. 4. Demonstration of C3 synergistic effect on PDAC cells.

Cell viability was evaluated using CellTiter Glo on PANC1, Mia PaC2, PDCL5 and PDCL15 cells based on the chosen drug concentrations and combinations (A). Combination index (CI) was calculated and plotted using CompuSyn program (B and C). The C3 combinations at IC50 level demonstrated a significant synergistic effect (D).

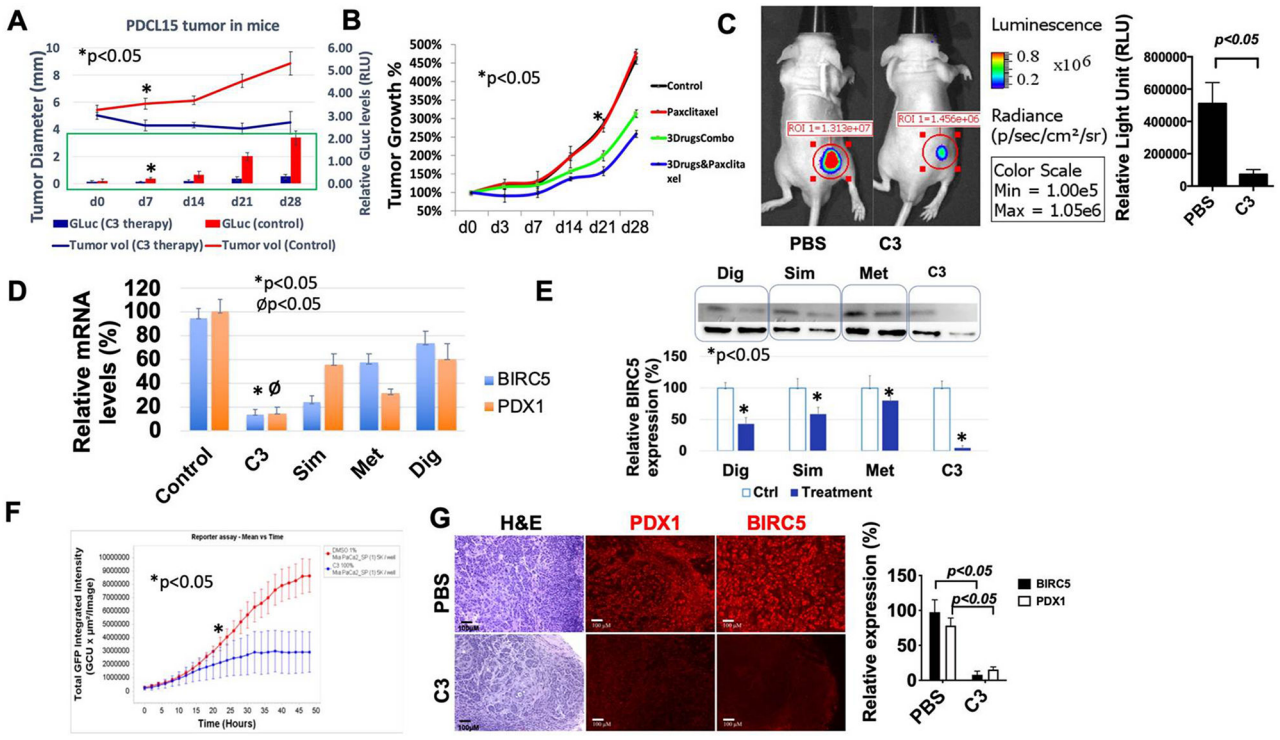


Fig. 5. C3 therapy suppressed tumor growth in PDCL15 xenograft mouse model.

CMV-GLuc-2A-TK stably transfected PDCL15 cells and Mia PaCa2 cells were subcutaneously implanted In nude mice. 2 weeks after inoculation, the mice received IP injection of C3. In PDCL15 model, there was no change in tumor volume (blue line) associated with an insignificant increase in serum GLuc levels (blue bar) over 28 d in the C3 treatment group (A), whereas tumor volume and serum GLuc levels (bars) consistently increased in the PBS control group (red color). PDCL15 tumor was also evaluated using bioluminescence imaging at 28 d after therapy showing significantly reduced signals in the C3 treatment group (B). In Mia PaCa2 model, C3 treatment resulted in significant decrease of tumor volume on day 21 ($P < 0.05$) and enhanced the paclitaxel's cytotoxicity to Mia PaCa2 tumor in mice ($p < 0.05$) (C). C3 significantly inhibited BIRC5 and PDX1 mRNA expression in PDCL5 cells (D) and significantly decreased protein levels in PANC1 cells (E), as determined by QPCR and Western blot, respectively, which was most likely through suppression of BIRC5 promoter's activity (F) as determined by IncuCyte. C3 therapy for PDCL15 tumors showed significant inhibition of BIRC5 and PDX1 protein expression as determined by IHC (G).

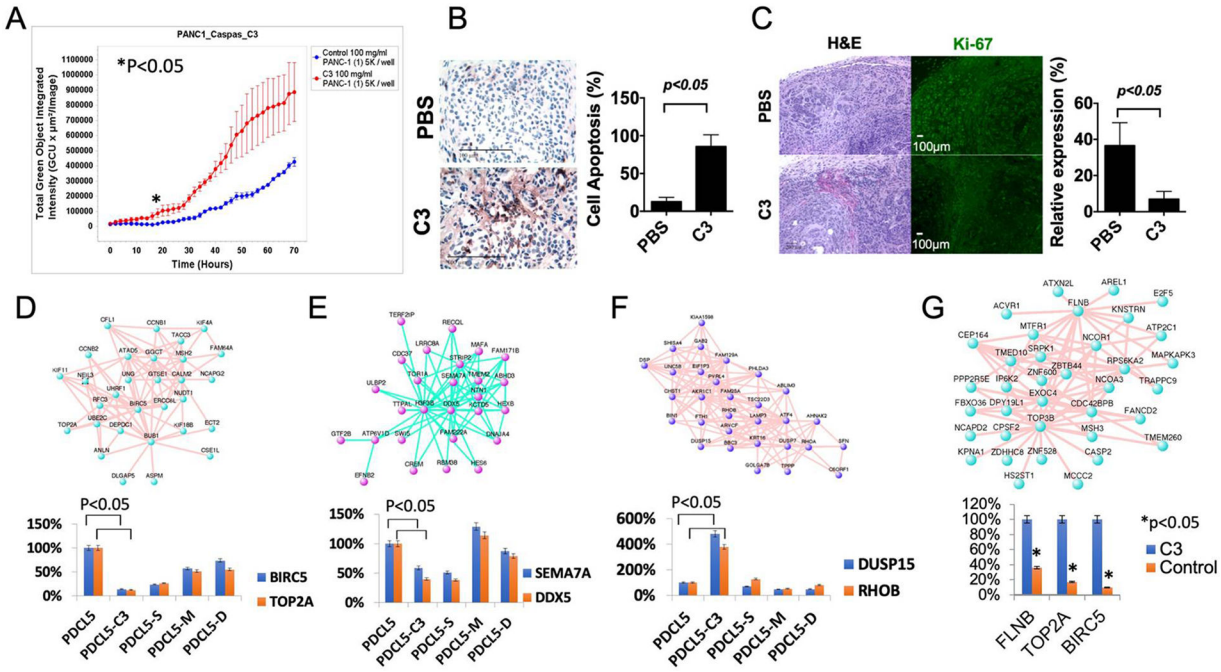


Fig. 6. Mechanisms of C3 suppression of PDAC.

Cell apoptosis in PDAC cells was dynamically evaluated by measuring caspase 3/7 in IncuCyte following 48 h. The curve showed C3 significantly induced cell apoptosis starting at 18 h (A). Significant increases of apoptosis in PDCL15 xenograft tumor was assessed using TUNEL assay after C3 treatment (B). C3 treatment also resulted in significant decrease of Ki67 expression in PDCL15 xenograft tumor *in vivo* (6C) using IF staining. WGCNA analysis identified three major networks based on the RNAseq data of C3 treated PDCL5 cells vs control, which included cell cycle network (D), ATP energy network and cell death network (E and F). Two genes from each network were chosen to be validated by QPCR (D, E and F, bar graph). WGCNA analysis was also applied to C3 treated PDCLI5 xenograft tumor specimens showing decrease of cell cycle enriched genes (G). Decrease of expression of FLNB, TOP2A and BIRC5 was validated by QPCR (G, bar graph).

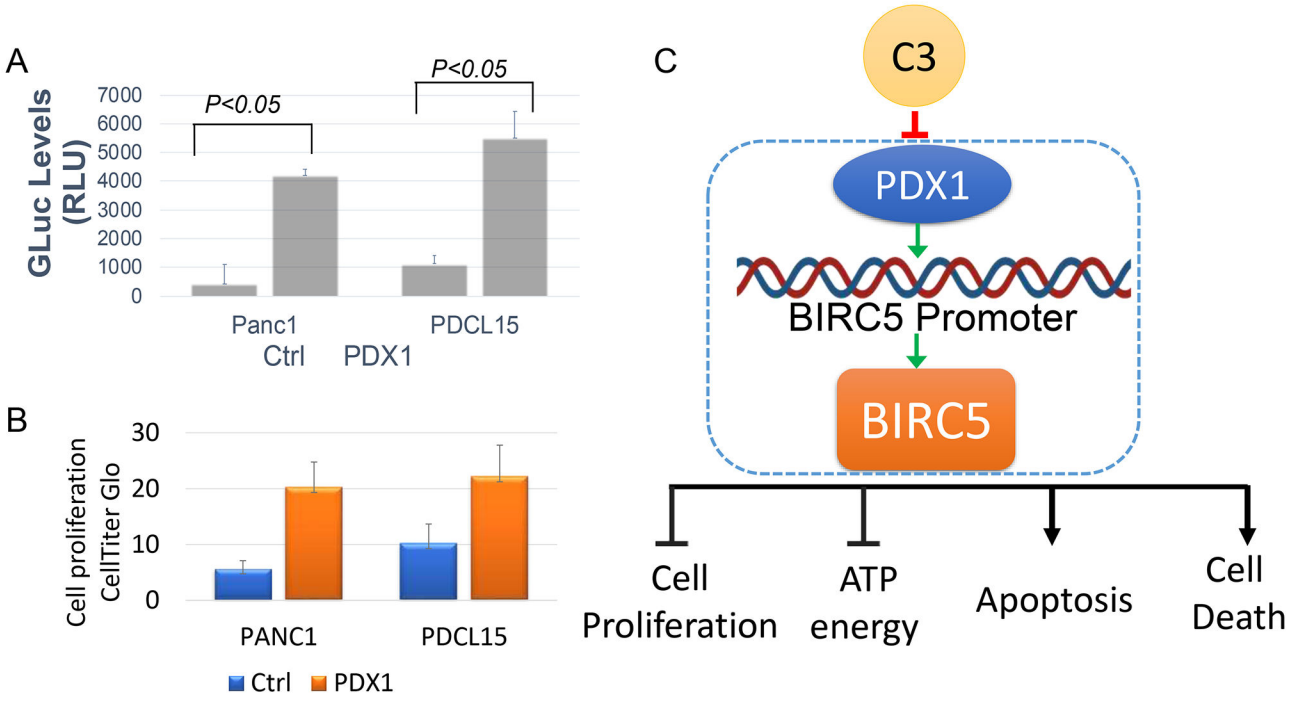


Fig. 7.

PDX1 activates the BIRC5 promoter. Co-transfection of PANC1 and PDCL15 cells with human PDX1 and BIRC5-SP-GLuc resulted in significant up-regulation of GLuc expression (A) and an increase of cell proliferation (B), indicating that PDX1 activated BIRC5's promoter in both cell lines (A, $p < 0.05$), leading to decreased cell proliferation, increased cell apoptosis and cell death via reduction of cell cycle and ATP-related gene expression and enhancement of cell death genes (Fig. 7C).

Table 1

Criteria for selecting metformin, simvastatin and digoxin

1	Safe and minimally toxic oral medications (package inserts)
2	No toxic chemotherapy
3	Off patent, generic, inexpensive
4	Patients are already taking the combination with minimal toxicity
5	Each drug targets separate pathways, but are all are antimetabolism drugs
6	Extensive literature on all 3 drugs and cancer therapy
7	This 3-drug combination is in use for chronic illnesses in thousands of patients

Table 2

in vitro and *in vivo* doses of simvastatin, digoxin and metformin used in current and other studies

Drugs	In vitro concentration	Used in other studies	In vivo dose	Lethal Dose (LD50)	Used in other studies
Simvastatin	4 μM	250 nM ~ 32 μM	20 mg/kg	798 mg/kg	20 mg/kg
Digoxin	50 nM	10 nM ~ 500 nM	2 mg/kg	4 mg/kg	2 mg/kg
Metformin	500 μM	300 μM ~ 40 mM	100 mg/kg	477 mg/kg	50 ~ 250 mg/kg