ANTICANCER RESEARCH *42*: 2835-2845 (2022)
doi:10.21873/anticanres.15765

Isoprenylated Coumarin Exhibits Anti-proliferative Effects in Pancreatic Cancer Cells Under Nutrient Starvation by Inhibiting Autophagy

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Abstract. *Background/Aim: Due to the lack of early detection methods and effective treatments, pancreatic cancer has one of the lowest five-year survival rates among all cancers. We have previously identified novel isoprenylated coumarin compounds that exhibit preferential cytotoxicity against pancreatic adenocarcinoma cell line PANC-1 exclusively under glucose deprivation conditions. Materials and Methods: Using cell cytotoxicity assays, we investigated the anti-proliferative mechanism of our most potent isoprenylated coumarin compound of the series, DCM-MJ-I-21, with respect to time, against two other pancreatic cancer cell lines, BxPC-3 and Capan-2. We used western blotting to quantify the autophagic flux influenced by our compound, autophagy inducers (starvation and Rapamycin), and autophagy inhibitors (chloroquine and wortmannin). Results: We observed a clear dependence on glucose in DCM-MJ-I-21 in BxPC-3 and Capan-2 pancreatic cancer cell lines, suggesting that our compound targets a pathway shared by these cancer cell lines when glycolysis is not an option for survival. Our lead compound increased the conversion of LC3-I to LC3-II in PANC-1, similar to the effect of chloroquine, an autophagy inhibitor. In addition, Spautin-1, another autophagy inhibitor, showed almost the same anti-proliferative activities at the same concentration under nutrient-deprived conditions as our lead compound in both 2D and 3D cell cultures. Conclusion: Our lead isoprenylated coumarin compound induces selective pancreatic cancer cell death under nutrient-deprived conditions through inhibition of autophagy, potentially providing insights into new therapeutic options.*

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Key Words: Novel anti-pancreatic cancer agent, coumarin derivative, selective cytotoxicity, autophagy.

Pancreatic cancer is one of the most aggressive and lethal solid tissue malignancies, with a 11% 5-year relative survival rate (1). Despite significant research efforts in the area of pancreatic ductal adenocarcinoma (PDAC), few new therapeutic strategies have emerged that have only marginally improved the survival and quality of life of PDAC patients. Reasons for the lack of transformative therapies include socioeconomic considerations, a lack of reliable early diagnostic tools leading to presentation at a late stage, the existence of multiple mechanisms of drug resistance, and a pathophysiology dictated by extreme hypovascularity (2-7).

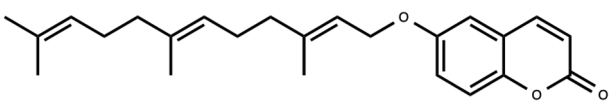
Currently, there are no reliable diagnostic tools for early detection of PDAC. The initial symptoms are similar to multiple common diseases, like peptic ulcer and gallbladder disease, which frequently lead to misdiagnosis in the early stages of the disease (8). The US Preventive Screening Task Force assigned screening for PDAC a grade 'D', meaning that early detection is either not helpful or that the potential harms of screening outweigh the potential benefits (9, 10). While the new techniques being developed for screening focus on biomarkers in the blood, proteins, microRNAs and more, they have not been widely studied yet or are not very reliable (11). Only 20% of patients have resectable tumors, and even then, there is an 80% chance of relapse (6, 7). This is concerning, since surgery is the only treatment that can potentially cure PDAC.

Furthermore, PDAC involves a complex interplay of systemic, cellular, and immune co-morbidities with multiple adaptations to the tumor environment that confer resistance to a variety of treatments. For instance, it has been demonstrated that PDAC metastasizes exceptionally early through micrometastases into neighboring tissues like the liver, lungs, and peritoneum,

which significantly limits the effectiveness of localized therapies such as surgery and radiation (12). An increasing number of reports identify the stroma, which can constitute 80% of the tumor, as an important factor in drug resistance of PDAC. Specifically, the attendant paucity of stromal vascularization and unusual desmoplastic cytology prevents the efficient delivery of anti-cancer drugs, while supporting immunosuppression within the tumor microenvironment, therefore aiding cancer growth (13). Desmoplasia, the extensive fibrosis at the tumor site, creates a hypoxic microenvironment, which promotes the progression of PDAC by enhancing the function of anti-angiogenic factors. This also contributes to the resistance to chemotherapy (14). Additionally, genomic analyses indicate that PDAC cells harbor multiple oncogenic driver mutations that play a role in genomic instability, tumor heterogeneity, and alterations of cell metabolism (5). These factors substantially complicate therapeutic approaches to the disease, rendering single-agent treatments ineffective. Finally, pancreatic cancer cells themselves contribute to immuno-suppression by preventing T cell activation and by upregulating the regulatory immune cells (14). The combination of these factors makes PDAC one of the most aggressive and lethal malignancies with more than 60,000 estimated US cases in 2022, calling for the urgent development of novel therapeutic strategies (1).

A distinctive feature of pancreatic cancer cells is their tolerance to nutrient- and oxygen-deprivation through tumor progression. It has been reported that four human PDAC pancreatic cancer cell lines, including PANC-1, survived for 48 h in the absence of essential amino acids, glucose, and serum, whereas normal fibroblasts died within 24 h under the same conditions (15). Since normal tissues seldom encounter nutrient deprivation, the austerity of pancreatic cancer cells under nutrient-deprived conditions has huge potential as a novel and selective biochemical target for cancer therapy.

In 2006, the natural product angelmarin was isolated from the Japanese medicinal plant *Angelica pubescens* and found to exhibit toxicity specifically against the pancreatic adenocarcinoma cell line PANC-1, at a concentration of 0.01 μg/ml under nutrient-deprived conditions (16). Motivated by this highly sensitive and apparently specific biological activity, we initiated an effort towards the total synthesis of angelmarin, but also recognized that the active pharmacophore may not require the entire molecule. Hence, as we progressed through the synthesis, we screened intermediates and analogs accessible from these intermediates for related activity against PANC-1. Our initial studies in this area culminated in the discovery of a family of coumarins possessing anti-proliferative activity against PANC-1, exclusively under nutrient-deprived conditions (17). We conducted a structure-activity-relationship (SAR) study, which revealed that bulky hydrophobic alkoxy substituents at certain positions of the coumarin scaffold showed activity analogous to angelmarin. In particular, isoprenylated alkoxycoumarins showed very promising activity



DCM-MJ-I-21

Figure 1. *Isoprenylated coumarin lead compound (1) designated as DCM-MJ-I-21.*

(100% cell growth inhibition at 6.25 μM concentration for the most potent analog) (17).

Based upon the encouraging activity exhibited by these compounds, we expanded our SAR study to explore substitution at other positions around the coumarin ring. These studies revealed that 6-alkoxycoumarins afforded better potency than the corresponding 7-substituted compounds. The optimal compound identified from these studies (6-farnesyloxy coumarin, compound 1, Figure 1) exhibited an LC_{50} value of 4 μM against PANC-1 under nutrient-deprived conditions and also induced apoptosis-like morphological changes in PANC-1 cells after a 24 h incubation (18-20).

With a library of potent and scalable compounds demonstrating activity against PANC-1 exclusively under nutrient-deprived conditions, we next sought to identify the molecular mechanism of action of these compounds. We began by investigating the effect of various cell culture medium components on the anti-proliferative activity of our compounds. These studies revealed that the absence of glucose is critical for the bioactivity of our isoprenylated coumarin compounds (21). In this study, while glucose was plentiful, our lead compounds were not cytotoxic, but as glucose supplies diminished, our compounds became increasingly effective. This suggests that the efficacy of our compounds derives from inhibition of a rescue pathway that predominates as a strategy for cancer cell survival under increasing nutrient deprivation. The regulated degradation and recycling of cellular components known as autophagy is a strategy frequently used by cancer cells to survive under stress conditions.

It has long been established that cancer cells can engage alternate metabolic pathways, primarily utilizing glycolysis over oxidative phosphorylation when nutrients are in short supply. The regulated cytological degradation pathway known as autophagy has been demonstrated to be integral to the survival of PDAC cells such as PANC-1 in the presence of chemotherapeutic drugs such as gemcitabine and 5'-fluorouracil (22). Studies have shown that autophagy inhibitors chloroquine and wortmannin suppress PANC-1 growth and induce apoptosis, suggesting autophagy has an anti-apoptotic effect (23). Given the selective cytotoxic

activity of our isoprenylated coumarin compounds under nutrient starvation conditions, we hypothesized that our compounds are either inhibiting or inactivating an autophagy pathway in cancer cells, which would otherwise enable their survival. We suggested that turning off this pathway, results in apoptosis or cell death. To further investigate the hypothesis that our isoprenylated coumarin compounds function through inhibition of autophagy that would normally allow PDAC cells to survive prolonged starvation, we conducted western blot analysis to examine the expression of the autophagy marker protein LC3 (24-26). In addition, we compared the anti-proliferative activity of our most potent isoprenylated coumarin compound of the series, DCM-MJ-I-21, to a well-known specific autophagy inhibitor Spautin-1 (27) under nutrient-deprived and nutrient-rich conditions. The studies presented herein demonstrate that our lead isoprenylated coumarin derivative, DCM-MJ-I-21, is a promising autophagy inhibitor for the treatment of pancreatic cancer.

Materials and Methods

Synthesis, purification, and characterization of DCM-MJ-I-21. DCM-MJ-I-21 was prepared as previously described (18). Briefly, 6-hydroxycoumarin (7.0 mmol, Sigma-Aldrich, St. Louis, MO, USA) and 40 ml of anhydrous *N,N*-dimethylformamide (DMF, Sigma-Aldrich) were added into an oven-dried round bottom flask. The solution was cooled to 0˚C, and then sodium hydride (7.0 mmol of 60% mineral oil suspension, Sigma-Aldrich) was added and stirred at 0˚C for 30 min. Farnesyl bromide (16.6 mmol, Sigma-Aldrich) dissolved in 10 ml of DMF was cooled to 0˚C and added dropwise to the reaction flask. The mixture remained stirring under nitrogen, warmed to room temperature overnight, and concentrated in vacuum. DCM-MJ-I-21 was purified *via* Preparative Thin Layer Chromatography (7:3 hexane:ethyl acetate), and the eluting solution was 2% methanol in dichloromethane. The structure of DCM-MJ-I-21 was confirmed by High Resolution Mass Spectrometry (70-VSE mass spectrometer at the University of Illinois), Hydrogen Nuclear Magnetic Resonance (^{1}H-NMR), and Carbon Nuclear Magnetic Resonance (^{13}C-NMR).

Preparation of medium. Human pancreatic cancer cell lines, PANC-1, BxPC-3, and Capan-2 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco Modified Eagle's Medium (DMEM), prepared with DMEM powder (Sigma-Aldrich), sodium bicarbonate solution (4.9%), antibiotic-antifungal solution (1×) (Sigma-Aldrich), and heat-inactivated fetal bovine serum (FBS, 10%) (Biowest, Riverside, MO, USA) in HPLC grade water, pH adjusted to 7-7.4, and filtered *via* a 0.2-μm Corning filter.

Nutrient-deprived medium (NDM) contained the following electrolytes and vitamin: $CaCl_2(2H_2O)$, 265 mg/l; $Fe(NO_3)(9H_2O)$, 0.1 mg/l; KCl, 400 mg/l; $MgSO_4(7H_2O)$, 200 mg/l; NaCl, 6400 mg/l; $NaHCO_3$, 700 mg/l; NaH_2PO_4, 125 mg/l; phenol red, 15 mg/L; HEPES buffer (25 mM, pH 7.4); and MEM vitamin solution (1×/l) (Life Technologies, Inc., Rockville, MD, USA). For nutrient-rich medium (NRM), additional nutrients were supplemented: FBS, 10%; D-glucose, 1,000 mg/l; L-glutamine, 2 mM; MEM amino acids solution and MEM nonessential amino acids solution (Life Technologies), 2% and 1%, respectively. For special media combinations, the supplements were added at the same concentration as described for NRM condition. For media with dialyzed serum, dialyzed serum (Life Technologies) was added as a replacement of FBS (10%).

Cytotoxicity assays. PANC-1, BxPC-3, or Capan-2 were seeded in 96-well plates at a density of 23,000 cells per well in DMEM, at 37˚C, 5% CO_2 for 24 h. After cells were gently washed with PBS, desired media conditions and DCM-MJ-I-21 concentrations (diluted with 5.5% DMSO in NDM) were added. After 24 h or desired time of incubation (for the time-dependent study), treated cells were washed with PBS and assessed for cytotoxicity with 10% WST-8 cell counting reagent (Dojindo Molecular Technologies) in DMEM, following a 3-h incubation. Cytotoxicity was measured as absorbance by using a plate reader (SpectraMax® M3 from Molecular Devices) at 450 nm, and cell viability was calculated: % cell viability={[Abs_{test} – Abs_{blank}]/[$Abs_{control}$ – Abs_{blank}]}*100%. The blank or 100% cell death represented cells treated with 1% Triton X-100 (Sigma-Aldrich), and the 100% cell survival control represented cells not treated with DCM-MJ-I-21 for each medium condition. At least three replicate experiments were conducted for each condition, and similar results were obtained.

Spheroid cytotoxicity assays. PANC-1 cells were seeded in 96-well Perfecta3D Hanging Drop Plates at a density of 10,000 cells per well in DMEM, at 37˚C, 5% CO_2 for 72 h. The medium was changed every 24 h, and HPLC water was added to the tray reservoirs to maintain a humid environment. Spheroids were abstracted into 2D 96 well plates through centrifugation (300 × *g*, 2 min) and incubated for 24 h [at this time point, a 2D cytotoxicity assay was started as a control]. After cells were washed with PBS, desired media conditions and desired therapeutic agent (DCM-MJ-I-21, Spautin-1) concentrations (diluted with 5.5% DMSO in NDM) were added. After 24 h, treated cells were assessed for cytotoxicity with 10% AlamarBlue cell counting reagent (Invitrogen, Waltham, MA, USA) in DMEM, following a 9 h incubation. Cytotoxicity was measured as fluorescence (excitation between 530-560nm and an emission at 590 nm) with a plate reader (SpectraMax® M3 from Molecular Devices). Cell viability was calculated following the same method as the 2D Cytotoxicity assays. Cells not treated with any therapeutic agent for each medium condition were considered as the 100% cell survival control. At least three replicate experiments were conducted for each condition, and similar results were obtained.

Cell lysates. PANC-1 cells were seeded in 6-well plates at a density of 600,000 cells per well in DMEM, at 37˚C, 5% CO_2 for 24 h. After rinsing with PBS, cells were treated with plain medium, 10 μM DCM-MJ-I-21, 25 μM chloroquine (Sigma-Aldrich), or 1 μM wortmannin (Sigma-Aldrich) in NRM or NDM for 24 h or desired incubation time. Due to the short half-life of wortmannin, PANC-1 was incubated with wortmannin for 4 h, then the medium was removed, and cells incubated in fresh NRM or NDM for another 20 h. Lysing solution was freshly made by diluting 100X Halt™ Protease Inhibitor Cocktail (Thermo Scientific) in cold Pierce RIPA Buffer (Thermo Scientific). Cells were washed with cold PBS twice, lysed with 150 μl cold lysing buffer, and kept on ice for 5 min with occasion stirring for uniform spreading of the buffer. Each cell lysate was collected with a cell

scraper, transferred to a micro-centrifuge tube, and centrifuged with Eppendorf Centrifuge 5804R at 14,000 rpm for 15 min at 4˚C. The protein concentrations of lysate supernatants were measured with NanoDrop 2000 Spectrophotometer (Thermo Scientific), and lysates were stored at –20˚C.

Western blotting. Cell lysates containing equal amounts of protein were added with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), boiled for 5 min, and then loaded onto 4-20% or any kD Mini-PROTEAN® TGX gels (Bio-Rad). Precision Plus Protein WesternC Standards or Dual Color Standards (Bio-Rad) were used as standard for each gel. The running buffer for the TGX gels were 1× Tris/Glycine/SDS (Novex), and the gels were run at 140-180 V. Polyvinylidene difluoride (PVDF) membranes (Bio-Rad) were activated in methanol prior to transfer, and the gel was transferred at 50-70 V for 90 min in cold transferring buffer, made by dissolving a pack of BuPH Tris-Glycine SDS Buffer (Pierce) in 200 ml methanol and 800 ml deionized water. The membrane was washed with washing buffer twice (1× Tris-buffered saline, 0.1% Tween20), and then blocked in blocking buffer (2.5 g blocking reagent from Bio-Rad in 50 ml washing buffer) for 60 min at room temperature. The membrane was then incubated on a shaker with polyclonal LC3B/MAP1LC3B antibody (1:1,000, Novus Biologicals, Centennial, CO, USA) for 4 h, and GAPDH antibody (1:3,000, Rockland, Limerick, PA, USA) for 1-2 h, with a quick wash between each primary antibody. After a thorough wash, the membrane was incubated with Goat anti-Rabbit IgG (1:5,000, Thermo Scientific) for 1 h, and optionally StrepTactin-HRP Conjugate (1:10,000, Bio-Rad) for 2-4 h. After another thorough wash, the membrane was sprayed with Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Holliston, MA, USA) and left for 1 min. The blot was visualized with ChemiDoc™ MP Imaging System (Bio-Rad), and the density of each band was measured using Image Lab software.

Results

Time-dependent cell cytotoxicity study of isoprenylated coumarin lead compound (DCM-MJ-I-21) in NDM and NRM against pancreatic adenocarcinoma cell line PANC-1. PANC-1 cells were treated with 10 μM DCM-MJ-I-21 in NDM and NRM for 8, 18, 24, 48, and 72 h (Figure 2). We used a concentration of 10 μM because DCM-MJ-I-21 exhibited an LC_{50} value of 9 μM in NDM after 24 h incubation (Table I). In this study, the survival of PANC-1 cells in NRM after 8 h incubation was defined as 100% survival reference. The exposure of PANC-1 cells to 10 μM DCM-MJ-I-21 in NDM demonstrated anti-proliferative activity after 8 h and induced 50% cell death after 24 h (Figure 2). PANC-1 cells reached 100% cell death in NDM after 48 h of incubation, regardless of treatment with DCM-MJ-I-21. In NRM, DCM-MJ-I-21 did not exhibit any anti-proliferative effect against PANC-1 until 72 h of incubation, when there was a sudden drop in cell survival, and a more pronounced drop with the presence of DCM-MJ-I-21 (Figure 2). This was due to the depletion of nutrients in the media after 48 h, thus NRM behaved NDM-like and the selective cytotoxicity of DCM-MJ-I-21 became noticeable. With media change after 24 and 48 h incubations to sustain enough nutrients, we were able to prevent such a survival drop and the cytotoxic effect of DCM-MJ-I-21 (data not shown).

Effect of cell culture media components on the selective cytotoxicity of DCM-MJ-I-21 in other pancreatic cancer cell lines. Since we previously demonstrated that both BxPC-3 and Capan-2 cell lines have similar selective sensitivity to DCM-MJ-I-21 to PANC-1 (21, Table I) under one single cell culture media condition, in this study we conducted a detailed investigation on the effect of the various cell culture media components against BxPC-3 and Capan-2. To this end, we tested DCM-MJ-I-21 *in vitro* against BxPC-3 and Capan-2 under 11 medium conditions (NRM and NDM as references, and different combinations of amino acids, glucose, serum, and dialyzed serum) to identify the key cell culture component(s) linked to the activity of our lead compound. The survival of cancer cells was examined after 24 h of incubation with DCM-MJ-I-21 by using the WST-8 cell counting reagent. Figure 3 summarizes the cell death and survival of all three pancreatic cancer cells lines treated with 100 μM DCM-MJ-I-21 in various cell culture media conditions, as well as the glucose concentration on a log scale in each medium. Survival of cells not treated with DCM-MJ-I-21 was defined as 100% cell survival for each medium condition, and cell survival of cells treated with 1% Triton X-100 was defined as 100% cell death. Similar to PANC-1, BxPC-3 and Capan-2 exhibited selective cytotoxicity under glucose deprivation. Nonetheless, Capan-2 was more sensitive to low glucose concentration in medium, as highlighted by its performance under NDM+Dia Ser and NDM+Dia Ser+AA conditions. Such difference in sensitivity might result from the intrinsic differences in the cell characteristics of these three cancer cell lines.

Autophagic flux detection with western blot analysis. Next, we examined the mechanisms by which our lead isoprenylated coumarin compound induces cytotoxicity in a nutrient-deprived environment. Microtubule-associated protein 1 light chain 3 was used to detect the autophagic flux in PANC-1 cells. Starvation and Rapamycin (Figure 4A) are autophagy inducers (22). Two well-characterized autophagy inhibitors, chloroquine (Figure 4B), which blocks the degradation of autophagosomes, and wortmannin (Figure 4C), which blocks the formation of autophagosomes, were used in this study as references (22). Cell lysates for western blotting were prepared by treating PANC-1 cells with plain media, 10 μM DCM-MJ-I-21, 25 μM chloroquine, or 1 μM wortmannin under both NDM and NRM for 24 h. Due to the short half-life of wortmannin, PANC-1 cells were incubated with wortmannin for 4 h, and then in either NRM or NDM for

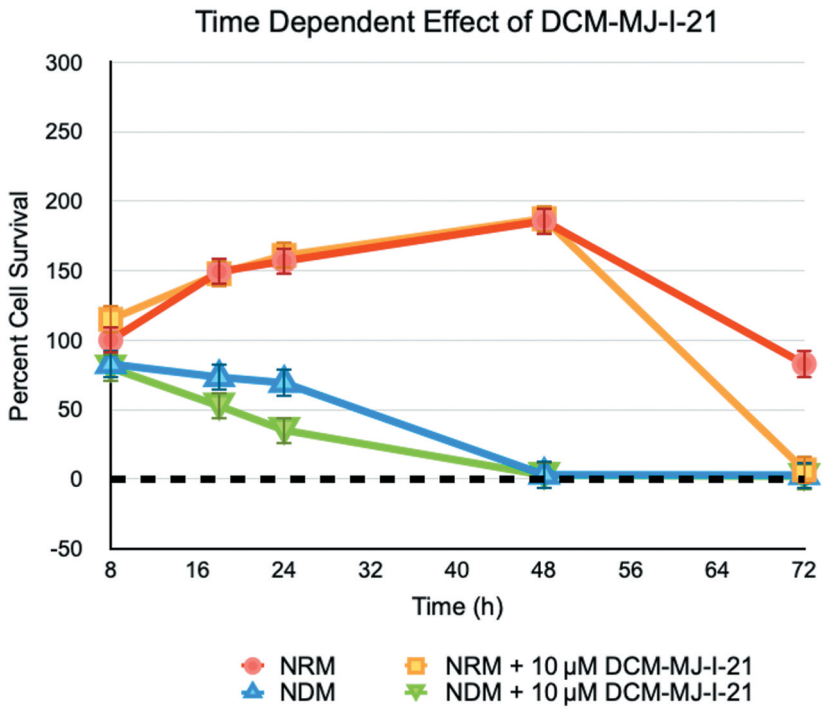


Figure 2. *Survival of PANC-1 cells under nutrient-deprived medium (blue) and nutrient-rich medium (red), and PANC-1 cells treated with 10 μM compound DCM-MJ-I-21 under nutrient-deprived medium (green) and nutrient-rich medium (orange) after 8-, 16-, 24-, 48-, and 72-h incubations. The survival of PANC-1 not treated with DCM-MJ-I-21 under nutrient-rich conditions after 8 h of incubation was defined as 100% survival reference. Values represent means±SEM, n=3. Replicate experiments were performed, and similar values were obtained. NDM: Nutrient-deprived medium; NRM: nutrient-rich medium.*

another 20 h. Western blotting (Figure 4D) showed that treatment of PANC-1 cells with chloroquine under both NRM and NDM resulted in an accumulation of LC3-II; however, treatment with wortmannin under both medium conditions decreased the levels of LC3-II. PANC-1 cells under NDM also showed an increase in the conversion of LC3-I to LC3-II compared to those under NRM, suggesting the induction of autophagy by starvation (Table II). Expression levels of LC3-I and LC3-II in PANC-1 were not significantly different than those after the 24-h treatment of DCM-MJ-I-21 under NRM (Figure 4D), consistent with prior results that DCM-MJ-I-21 shows no cytotoxicity under NRM. In contrast, treatment of PANC-1 cells with DCM-MJ-I-21 under NDM for 24 h increased the conversion of LC3-I to LC3-II (from 1.57 to 1.88, Table II), possibly suggesting an inhibition of autophagy in the same manner as chloroquine. This potential inhibition of autophagy under nutrient-deprived conditions agrees with the preferential cytotoxicity of DCM-MJ-I-21 exclusively under glucose deprivation conditions.

Time-dependent autophagy induced by DCM-MJ-I-21 and chloroquine. PANC-1 cells were treated with plain medium, 10 μM DCM-MJ-I-21, or 25 μM chloroquine under both NRM and NDM for 3, 7, 12, and 24 h, and western blotting was used to detect the expression levels of LC3-I and LC3-II (Figure 5). Under NRM, the levels of LC3 proteins did not change after treatment with DCM-MJ-I-21; however, treatment with chloroquine led to an accumulation of LC3-II with a longer time of incubation. Under NDM, both DCM-MJ-I-21 and chloroquine led to accumulation of LC3-II over time, further evidence suggesting that DCM-MJ-I-21 could behave similar to chloroquine in inhibiting autophagy under nutrient-deprived conditions.

Table I. *LC_{50} values of DCM-MJ-I-21 under nutrient-deprived medium (NDM) and nutrient-rich medium (NRM) against three pancreatic cancer cell lines: PANC-1, BxPC-3, and Capan-2.*

	LC_{50} (μM) in NDM	LC_{50} (μM) in NRM
PANC-1	9	>100
BxPC-3	5	>100
Capan-2	5	>100

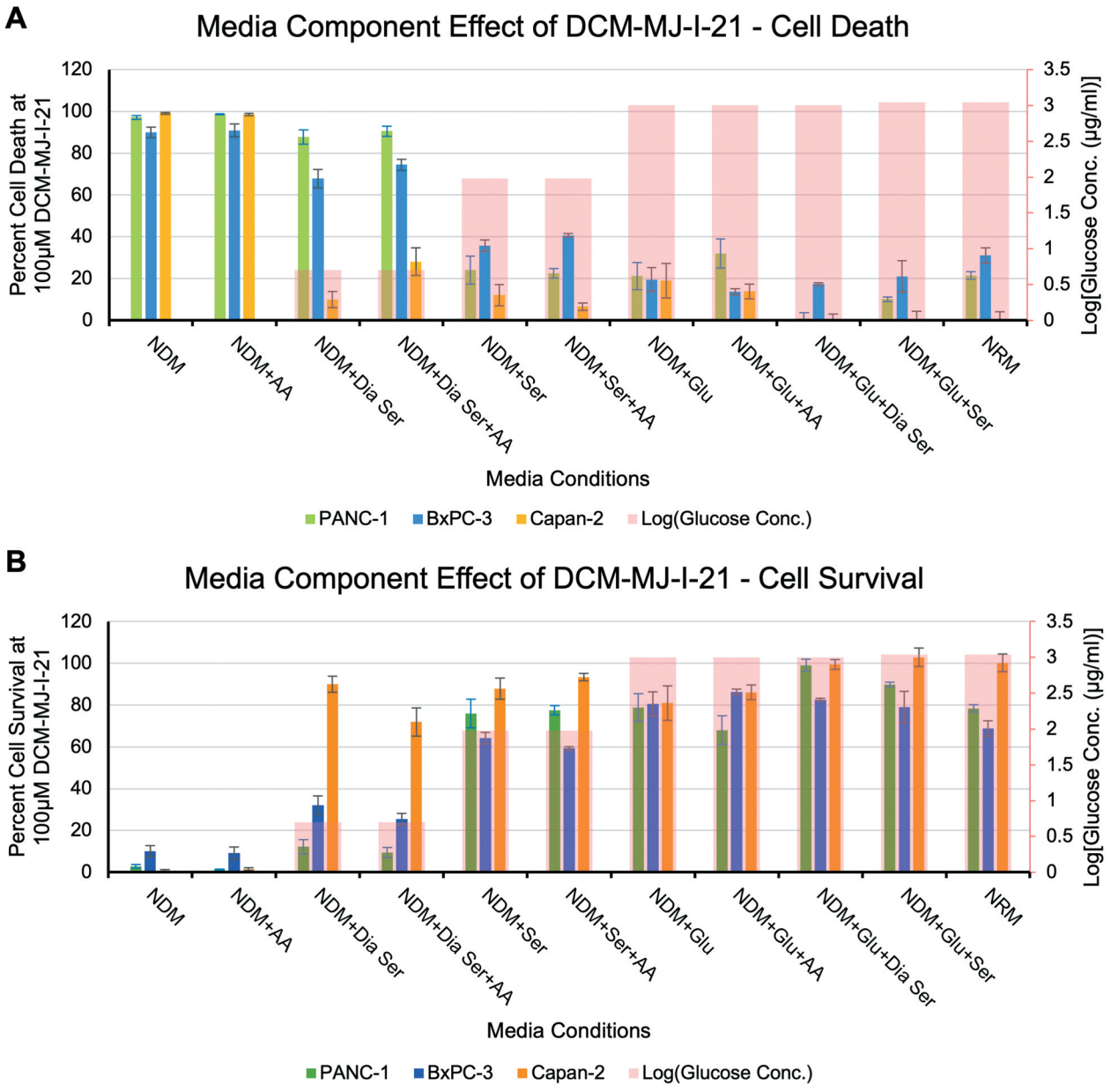


Figure 3. *Percent PANC-1 (green), BxPC-3 (blue), and Capan-2 (orange) cell death (A) and cell survival (B) upon exposure for 24 h to 100 μM of DCM-MJ-I-21 in cell culture media conditions of various nutrient components: glucose (Glu), amino acid (AA), serum (Ser), and dialyzed serum (Dia Ser). All results are means±SEM, n=3. The final concentration of glucose (μg/ml) in each medium condition, plotted on a log scale (pink), is overlaid with the corresponding viability histogram.*

Cytotoxicity comparison with Spautin-1 in 2D and 3D cell cultures. Spautin-1 (Figure 6A), a well-known specific inhibitor of autophagy, causes an increase in proteasomal degradation of class III PI3 kinase complexes (27). We next compared the anti-proliferative activity of our lead compound to the autophagy inhibitor Spautin-1, under NDM and NRM after a 24 h treatment period, and to our delight, we observed almost identical effects (Figure 6B and D). Based on the similar growth inhibition potency and selectivity of DCM-MJ-I-21 and Spautin-1 against PANC-1 in dispersed 2D cell monolayers, we compared the cytotoxicity of DCM-MJ-I-21 and Spautin-1 against PANC-1 3D cell cultures. To this end, we compared the cell growth inhibition activity against PANC-1 spheroids using a hanging-drop technique. These studies showed that DCM-MJ-I-21 retains the preferential activity seen under

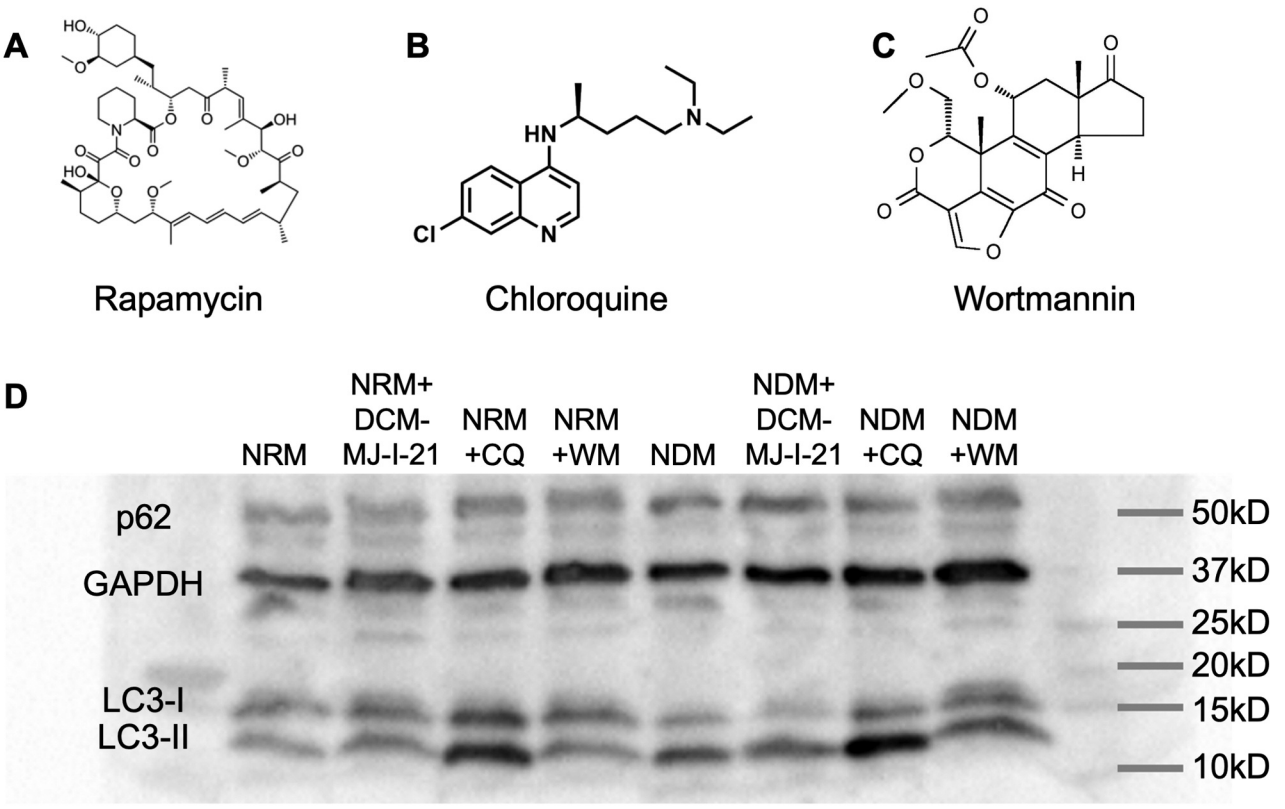


Figure 4. *Starvation and rapamycin (Rapa, A) function as autophagy inducers, chloroquine (CQ, B) as autophagy inhibitor by blocking degradation of autophagosomes, and wortmannin (WM, C) as autophagy inhibitor by blocking formation of autophagosomes (22). (D) Effects of DCM-MJ-I-21 and autophagy inhibitors on the expression levels of LC3-I, LC3-II, and p62 in PANC-1. Western blot analysis was performed on cell lysates after treating PANC-1 cells with plain media, 10 μM DCM-MJ-I-21, 25 μM CQ, or 1 μM WM under both NDM and NRM for 24 h. NDM: Nutrient-deprived medium; NRM: nutrient-rich medium.*

Table II. *The band densities of the western blot shown in Figure 4D as measured with Image Lab.*

	NRM	NRM+DCM-MJ-I-21	NRM+CQ	NRM+WM	NDM	NDM+DCM-MJ-I-21	NDM+CQ	NDM+WM
GAPDH	20.222,30	24.122,30	26.042,40	27.575,10	24.258,20	28.565,20	30.864,40	33.770,40
LC3-I	15.156,20	17.303,50	21.028,70	18.593	11.673,60	10.262,80	17.450,80	18.893,20
LC3-I ratio to GAPDH	0.75	0.72	0.81	0.67	0.48	0.36	0.57	0.56
LC3-II	11.965,30	15.915,90	26.158,10	15.274,80	18.279,80	19.258	30.927,80	17.899,80
LC3-II ratio to GAPDH	0.59	0.66	1.00	0.55	0.75	0.67	1.00	0.53
LC3-II/LC3-I	0.79	0.92	1.24	0.82	1.57	1.88	1.77	0.95

nutrient deprivation in 3D conditions (Figure 6E). Similarly, 3D cell cytotoxicity assays performed with Spautin-1 demonstrated little to no cell death under nutrient-rich conditions (Figure 6C), but a dramatic drop in percent cell survival was observed under nutrient-deprived conditions after treatment with 6.25 μM Spautin-1 (Figure 6B and C) in both assay types.

Discussion

Our previous studies showed that our lead compound DCM-MJ-I-21 (Figure 1) induces selective cytotoxicity against PANC-1 cells under glucose deprivation (17, 18, 21), suggesting that this compound targets a pathway when glycolysis is not an option to sustain cancer cell survival. Hoping to gain insight

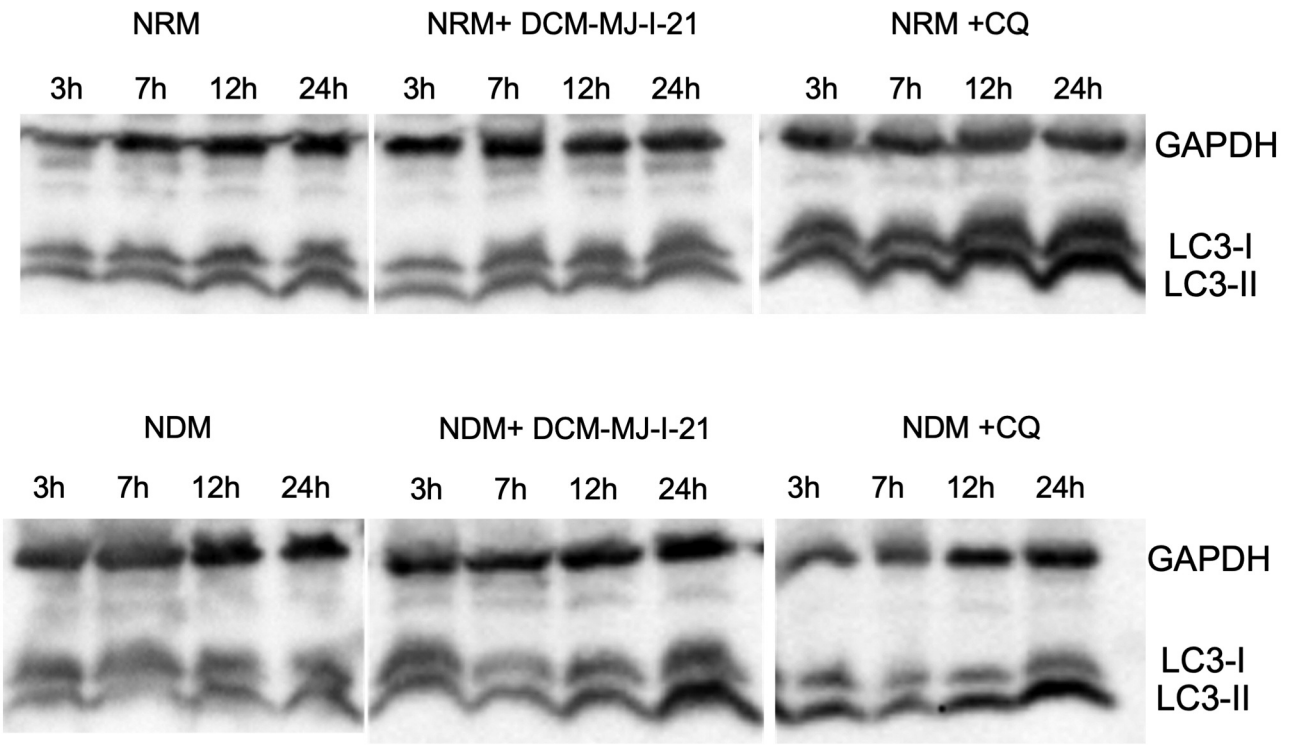


Figure 5. *Western blot analysis was performed on cell lysates after treating PANC-1 cells with plain media, 10 μM DCM-MJ-I-21, or 25 μM CQ under both NRM and NDM for 3, 7, 12, and 24 h. CQ: Chloroquine; NRM: nutrient-rich medium; NDM: nutrient-deprived medium.*

into its anti-proliferative mechanism with respect to time, we treated cells with our compound in NDM and NRM for multiple time periods (8, 18, 24, 48, and 72 h) instead of the previously studied single time 24-h treatment period. DCM-MJ-I-21 exhibited anti-proliferative activities at 8-48 h of exposure in NDM. To further explore whether DCM-MJ-I-21 induced PANC-1 cell death through a pathway unique to pancreatic cancer cells, we investigated the anti-proliferative activity of DCM-MJ-I-21 and nutrient deprivation in two other pancreatic cancer cell lines, BxPC-3 and Capan-2, and observed a similar dependency on glucose concentration as that observed with PANC-1 cells. The similar trend of higher glucose concentration and lower cell death in all these pancreatic cancer cell lines suggested that DCM-MJ-I-21 targets a pathway shared by most types of pancreatic cancer cells.

We have previously hypothesized that DCM-MJ-I-21 exerts its action *via* the inhibition of autophagy, the recycling of nonessential cellular components for cancer cell survival under nutrient deprivation (21). To further evaluate this hypothesis, we measured the autophagy flux with western blotting, which showed that DCM-MJ-I-21 treatment in PANC-1 under NDM increased the conversion of LC3-I to LC3-II, but not under NRM. This provided us the first evidence that the inhibition of autophagy could explain the preferential cytotoxicity of our compound. Given the bicyclic structural similarity of DCM-MJ-I-21 (Figure 1) and chloroquine (Figure 4B), we performed a closer comparison of the two compounds at different treatment time points. The similar autophagy flux of the two compounds under NDM further suggested that DCM-MJ-I-21 could inhibit autophagy similar to chloroquine.

While we were searching for other autophagy inhibitors for additional comparative studies, we identified the potent small molecule Spautin-1, which is known for its autophagy inhibition mechanism. We compared the anti-proliferative activities of our compound and those of Spautin-1 under NDM and NRM in both 2D and 3D cell cultures. The very similar anti-proliferative activity patterns exhibited by DCM-MJ-I-21 and Spautin-1 in nutrient-deprived and nutrient-rich conditions and in both 2D and 3D assays (Figure 6D and E) provide further support to the accrued evidence suggesting that DCM-MJ-I-21 functions as an autophagy inhibitor.

In conclusion, our studies show that our lead compound DCM-MJ-I-21 increases the conversion of LC3-I to LC3-II in PANC-1 cells, similar to the effect of chloroquine, a well-known autophagy inhibitor. In addition, the anti-proliferative effect of our lead compound was found to be almost identical to that of the potent small molecule specific autophagy inhibitor Spautin-1 under nutrient-deprived and

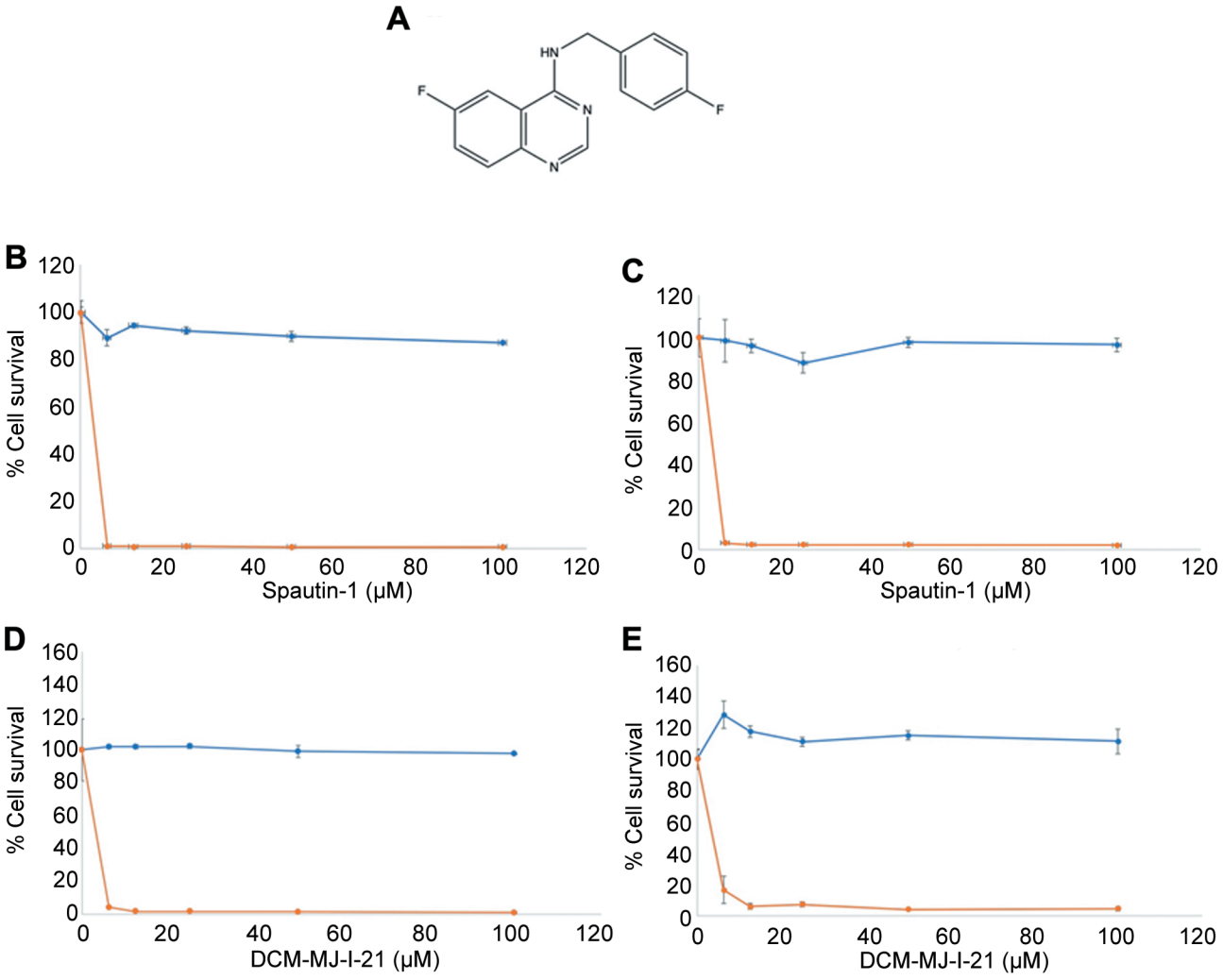


Figure 6. *Comparison of Spautin-1 and DCM-MJ-I-21. (A) Structure of Spautin-1, an autophagy inhibitor that increases degradation of beclin-1. Survival of PANC-1 cells in 2D (B) and 3D (C) cell culture under nutrient deprived medium (orange) and nutrient rich medium (blue) after 24 h incubation with Spautin-1. Survival of PANC-1 cells in 2D (D) and 3D (E) cell culture under nutrient deprived medium and nutrient rich medium after 24 h incubation with DCM-MJ-I-21. The selected therapeutic agent was administered at six different concentrations (100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 0 μM). Error bars represent standard error.*

nutrient-rich conditions after a 24 h treatment period against PANC-1 in both 2D and 3D cell cultures. Combined, these studies support the hypothesis that DCM-MJ-I-21 functions through inhibition of autophagy that would normally allow PDAC cells to survive prolonged starvation, and provides a promising lead compound for developing a class of autophagy inhibitors as potential anticancer agents.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

Wet laboratory experiments were performed by R.Z., E.K., Y.W., and J.Z. A.W. and D.M. supervised the work. All Authors contributed to the editing of the manuscript.

Acknowledgements

This work was supported by a faculty award grant from Wellesley College, MA, USA (Grant/Award Number: FF24181). The Authors wish to thank Milica Markovic, Elisa Wang and Paige Hauke for their early work on the spheroid cytotoxicity assays.

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Received March 23, 2022
Revised April 18, 2022
Accepted April 21, 2022