Investigation of the Antiproliferative Mechanism of Novel Isoprenylated Coumarin Compounds Against Pancreatic Cancer Cells

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Investigation of the Antiproliferative Mechanism of Novel Isoprenylated Coumarin Compounds Against Pancreatic Cancer Cells

Ronghao Zhou

Submitted in Partial Fulfillment of the Prerequisite for Honors in Chemistry under the advisement of Dr. Dora Carrico-Moniz.

December, 2016

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I have not been good at expressing my feelings, but at this time of my life, realizing the day of me leaving Wellesley is approaching, I feel I have so many to say, so many to thank, and so many to miss. I have been so fortunate to receive so much from Wellesley, and all these memories will stay with me forever.

I have to express my most sincere gratitude to my advisor, Prof. Dora Carrico-Moniz for your all-time guidance, physical and moral supports, and cares. I am so lucky to have joined DCM lab. You always think the best for me, protect me, and guide me to become a better person.

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And thank you so much, Prof. Don Elmore for being in my thesis committee and giving me advice. I have really enjoyed taking the biochemistry classes with you.

Lastly, many thanks to my lab partners and friends for your accompany. Lots of thanks to Frieda Zhang’15, I have learned so much from you and you will always be my role model. Thanks to Yin Wang’16 for being such a wonderful lab partner and sharing your food with me. Thank you everyone in the DCM lab!

I could not complete with thesis without anyone’s help and support! Thank you so much all! This thesis will be the best graduation gift I will ever received!
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ABSTRACT

Pancreatic cancer is one of the most devastating forms of human cancer, and due to the lack of effective treatments, it has one of the lowest five-year survival rates among all cancers. Angelmarin, isolated from the medicinal plant *Angelica pubescens*, has showed anti-pancreatic cancer activity under nutrient-deprived conditions. Recent works in our laboratory have identified a novel isoprenylated coumarin compound 21, derived from angelmarin, to exhibit preferential cytotoxicity against pancreatic adenocarcinoma cell line PANC-1 exclusively under glucose deprivation, suggesting 21 targets a pathway when glycolysis is not an option for cancer cell survival. In this study, the anti-proliferative mechanism of 21 was investigated. In particular, 21 was found to increase the conversion of LC3-I to LC3-II in PANC-1, similar to the effect of chloroquine, an autophagy inhibitor, supporting the potential mechanism of 21 through chloroquine-like inhibition of autophagy. Furthermore, current results showed while chloroquine inhibits under all conditions, 21 inhibits autophagy only under nutrient-deprived conditions.
INTRODUCTION

1. Pancreatic Cancer

Statistics and Treatments for Pancreatic Cancer

Pancreatic ductal adenocarcinoma is one of the most devastating forms of human cancer. It is the third leading cause of cancer death in the United States. The incidence and death rates for pancreatic cancer are still increasing; in 2016, the American Cancer Society has estimated 53,070 new cases and 41,780 deaths\(^1\). Moreover, pancreatic cancer has the highest mortality rate among all major cancers, and is also known for its early metastasis and aggressive invasion of surrounding tissues. The overall 5-year survival rate is 12-14% for patients with localized disease, and merely 1% for patients with distant metastasis\(^2\). It is also one of the few cancers for which survival has not improved significantly over the past 40 years. Currently, there is no standard diagnostic instrument or established early detection method for pancreatic cancer\(^3\). If diagnosed early, surgical removal of the tumor provides the best chance at a definitive cure, yet the 5-year survival rate merely increases to 27%. In addition, only 10-20% of patients are diagnosed at a stage that is amenable to resection\(^4\). Due to the lack of recognizable symptoms and early detection methods, patients are usually diagnosed at late tumor stages without surgical therapy options\(^5\).

Chemotherapy or chemotherapy with radiation is usually the alternative for patients whose tumor could not be removed surgically. However, pancreatic cancer is highly resistant to conventional chemotherapies, including paclitaxel, 5-fluorouracil, leucovorin, doxorubicin, gemcitabine, Cisplatin, and platinum-based drugs\(^6-8\). The lack of effective clinical treatments for pancreatic cancer makes it one of the cancers with the lowest five-year survival rates, and has the
highest global mortality rate of nearly 98%. Therefore, it remains urgent to develop novel and effective therapies against pancreatic cancer.

**Pancreatic Cancer Cell**

Pancreatic cancer cells have been found to possess tolerance for starvation, which might be a reason to explain the aggressiveness of pancreatic cancer. A study in 2000 reported four pancreatic cancer cell lines, PANC-1, ASPC-1, BxPC-1, and KP-3 survived under extreme nutrient deprivation (deprivation of serum, glucose, and amino acids) for 48h, while normal human fibroblasts would die within 24h. One possible molecular mechanism for such tolerance, as proposed by the same study, is closely associated with the high expression of PKB/Akt, which is a protein kinase that regulates cell survival and proliferation. The results suggested Akt could be stimulated by the deprivation of amino acids or glucose, or both.

Elimination of this starvation-resistance could become a new strategy for cancer therapy. Since the discovery of the tolerance of pancreatic cancer cells to nutrient deprivation, several research studies have aimed to investigate the key factors responsible for the tolerance. For instance, one study compared the expression of Hypoxia-inducible Factor-1α protein in pancreatic cancer cells to that in other cancer cells, and found cells with the constitutive expression of HIF-1α were more resistant to apoptosis induced by glucose deprivation. Another study demonstrated the critical contribution of AMP-activated protein kinase to protect cancer cells from metabolic stresses under nutrient-deprived conditions. Furthermore, a more recent study showed the important role of GCN2-ATF4 pathway in cancer cell tolerance of starvation; ATF4 is the transcription factor that regulates the genes involved in amino acid metabolism and
redox homeostasis, and it is upregulated by the initiation factor kinase GCN2, which could be activated by glucose or amino acid deprivation.

Recently, autophagy, the degradation and recycling of nonessential cellular components, has been shown to sustain pancreatic cancer PANC-1 cells under extreme nutrient deprivation\textsuperscript{13-14}. The Baek Lab has demonstrated that autophagy inhibitors, chloroquine and wortmannin, would suppress PANC-1 growth and induce apoptosis, suggesting autophagy has an anti-apoptotic effect\textsuperscript{13}. Furthermore, the Laukkarinen Lab has reported that autophagy has a cytoprotective effect against the anticancer drugs 5-fluorouracil and gemcitabine\textsuperscript{14}. In the studies described herein, we hypothesized that the preferential cytotoxicity of our lead compound exclusively under nutrient-deprived conditions resulted from the inhibition of autophagy.

2. Natural Products in Medicine and Angelmarin

\textit{Natural Products as Anticancer Agents}

Nature has provided the largest treasures for drug discovery, and has inspired the development of modern medicines\textsuperscript{15-16}. The \textit{Compendium of Materia Medica}, written by Li Shizhen during the Ming Dynasty, has record of 1892 entries of natural plants, animals, and minerals that have medicinal properties, and it is highly regarded as the most comprehensive medical book in the history of Chinese traditional medicine\textsuperscript{17}. Youyou Tu, a Chinese woman doctor, won the 2015 Nobel Prize in Physiology or Medicine for her discoveries of artemisinin (\textbf{1}, Figure 1) from \textit{Artemisia annua} L. (Figure 1), as a novel therapy against Malaria\textsuperscript{18}. Other significant drugs developed from natural medicinal plants include reserpine, the antihypertensive agent from \textit{Rauwolfia serpentina}, ephedrine, the anti-asthma agent from \textit{Ephedra sinica}, and tubocurarine, the muscle relaxant from \textit{Chondrondendron} and \textit{Curarea} species\textsuperscript{19}. 
For treatment of cancer, natural products have also played an important role. For instance, Vinca alkaloids are a subset of drugs derived from the pink periwinkle *Catharanthus roseus*, that are used as anti-cancer treatments\textsuperscript{20}. Paclitaxel (Taxol\textsuperscript{®}, 2, Figure 2) is another anticancer drug isolated from the bark of the Pacific yew tree *Taxus brevifolia*, and is now commonly used as chemotherapy against various types of cancer, including ovarian, breast, lung, and pancreatic cancers. Since the discovery of paclitaxel, analogues such as docetaxel (Taxotere\textsuperscript{®}, 3), Abrazane\textsuperscript{®}, and Cabazitaxel (Jevtana\textsuperscript{®}, 4) have been developed and approved with some significant clinical advantages\textsuperscript{19}.

**Figure 1.** Picture of *Artemisia annua* L. (Qinghao) and structure of Artemisinin (Qinghaosu). Pictures taken from Tu. 2011.

![Artemisinin (1)](image)

**Figure 2.** Structures of Paclitaxel, and its analogues Docetaxel and Cabazitaxel.
Natural products have provided inspirations for drug discovery and development. However, the limited quantities of natural products obtainable from their natural sources are insufficient for large-scale drug production. Therefore, after the initial discovery of a natural product with clinical properties, total synthesis of the natural product becomes the challenging task for organic chemists. Sometimes, analogues discovered in the process of synthesizing the natural product offer even better medical properties than the natural product itself. In our lab, we are interested in investigating structural analogues of the natural product angelmarin (5, Figure 3) as promising anticancer agents.

**Discovery of Angelmarin**

In 2006, a novel natural product, angelmarin (5, Figure 3), isolated from the root of the medicinal plant *Angelica pubescens*, was found to kill pancreatic cancer PANC-1 cells preferentially under nutrition starvation. Angelmarin is composed of a columbianetin core (blue, Figure 3) and \( p \)-hydroxycinnamoyl tail (red, Figure 3). At a concentration of 0.01 \( \mu \)g/mL, angelmarin induced 100% cytotoxicity against PANC-1 cells after 24h of starvation, and when the concentration increased to 10\( \mu \)g/mL, 100% cell death was observed within 6h. On the other hand, the structural components of angelmarin, columbianetin and \( p \)-hydroxycinnamic acid were both inactive in nutrient-rich and nutrient-deprived conditions, suggesting the significance of the special structure of angelmarin to enable cytotoxic activity\(^{21}\).

![Angelmarin Structure](image)

**Figure 3.** Structure of Angelmarin, and its structure components columbianetin core (blue) and \( p \)-hydroxycinnamoyl tail (red)
3. Structure-Activity Relationship (SAR) Studies of Coumarin Derivatives

*SAR Study of Hydroxycoumarin Derivatives*

Structure-Activity Relationship (SAR) is the study of the relationship between the compound’s molecular structure and its biological activity. The analysis of SAR allows scientists not only to pinpoint the chemical groups responsible for the target biological effect, but also to modify the compounds to achieve the greatest potency and least side effects.

The basic structure of Angelmarin is a coumarin (6, Figure 4) core. Coumarins are ubiquitous in a number of natural drugs that possess an extensive range of physiological activities, including anticancer, anti-viral, antifungal, anti-inflammatory, antioxidant, anticoagulant, antibacterial, antitubercular, and analgesic activities. More specifically, hydroxycoumarin derivatives have been found to show promising antitumor, anti-inflammatory, and anti-viral effects\(^{22}\).

To investigate the relationship between the structures of coumarin derivatives and their preferential cytotoxic activity against pancreatic cancer cells, our lab started the first SAR studies of simple hydroxycoumarin-based compounds (7-16, Table 1) as potential anticancer agents. The compounds were tested against PANC-1 cells under nutrient-deprived conditions. While compounds 7-11 didn’t show any appreciable cytotoxic activity, compound 12 induced 100% cell death at a high concentration of 200µM. Analysis of the 5-carbon (compound 13), 10-carbon (14), 15-carbon (15), and 20-carbon (16) isoprenyloxy derivatives, suggests the presence of
longer carbon chain tails or higher hydrophobicity correlates to higher cytotoxic activity. At the same time, the geranylgeranyl hydroxycoumarin derivative, compound 15, was discovered to exhibit the highest preferential cytotoxicity against PANC-1 at a concentration of 6.25µM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. for 100% death ND</th>
<th>Compound</th>
<th>Conc. for 100% death ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>&gt;200µM</td>
<td>8</td>
<td>&gt;200µM</td>
</tr>
<tr>
<td>9</td>
<td>&gt;200µM</td>
<td>10</td>
<td>&gt;200µM</td>
</tr>
<tr>
<td>11</td>
<td>&gt;200µM</td>
<td>12</td>
<td>200µM</td>
</tr>
<tr>
<td>13</td>
<td>100µM</td>
<td>14</td>
<td>25µM</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>6.25µM</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td>50µM</td>
</tr>
</tbody>
</table>

Table 1. *In vitro* cytotoxicity of hydroxycoumarin derivatives (7-16) against PANC-1 under nutrient-deprived (ND) condition within 24h. Conc. obtained from Devi et al., 2011.22
**SAR Study of Isoprenylated Coumarins**

After the discovery of the geranylgeranyl coumarin derivative (9, Table 1), our lab launched our second SAR studies on the relationship between the isoprenyl positioning as well as the tail length and the cytotoxic activity against pancreatic cancer cells. A library consisting of three series of isoprenylated coumarin derivatives with systematic variations in the substitution position on the coumarin scaffold (at 3-, 6-, and 7-positions, Figure 4) and the tail length (5-, 10-, and 15-carbon) was created\textsuperscript{23-25}. These ether compounds were prepared via Williamson ether synthesis with an isopropyl bromide and an alkoxycoumarin (Scheme 1)\textsuperscript{23}.

\[ \text{Br} \quad \text{Na} \quad \text{O} \quad \text{Coumarin} \rightarrow \quad \text{O} \quad \text{Coumarin} \]

**Scheme 1.** General synthetic scheme for preparation of isoprenylated coumarins.\textsuperscript{23}

All three compound series were tested *in vitro* against PANC-1 cells under nutrient-deprived (ND) and nutrient-rich (NR) conditions. The mean 50% lethal concentration (LC\textsubscript{50}) values after 24h exposure to the compounds are summarized in Table 2. All compounds were inactive under nutrient-rich conditions, and showed selective cytotoxicity only under nutrient-deprived conditions. In addition, within each series, the same trend was observed: increase in tail length corresponds with decrease in LC\textsubscript{50} values and increase in cytotoxic activity\textsuperscript{23}.

Among all isoprenylated coumarin derivatives tested, compound 21 displayed the greatest cytotoxicity under nutrient-deprived conditions with an LC\textsubscript{50} value as low as $4\mu\text{M}$\textsuperscript{23}. To better understand the preferential cytotoxicity and to investigate the potential cellular mechanism of our coumarin derivatives, we have picked compound 21 to be our lead compound for the future studies because of its highest potency.
<table>
<thead>
<tr>
<th>Series</th>
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<th>LC$_{50}$ (µM) ND</th>
<th>LC$_{50}$ (µM) NR</th>
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</thead>
<tbody>
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<td>1</td>
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<td>&gt;100</td>
</tr>
<tr>
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</tr>
<tr>
<td>22</td>
<td><img src="image9" alt="Compound 22" /></td>
<td>18</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table 2. *In vitro* cytotoxicity of isoprenylated coumarin derivatives (13-14, 16-22) against PANC-1 under nutrient-deprived (ND) and nutrient-rich (NR) conditions within 24h. LC$_{50}$ values obtained from Jun *et al.*, 2014.$^{23}$
4. Preferential Cytotoxicity of Compound 21 Against Other Pancreatic Cancer Cell Lines

The anti-proliferative activity of lead compound 21 was also investigated in vitro against two other pancreatic cancer cell lines, BxPC-3 and Capan-2 (Figure 5). Similar to PANC-1, both cell lines exhibited cytotoxicity to 21 preferentially under nutrient-deprived conditions with LC_{50} values of 5\mu M for both cell lines (Table 3), while no cytotoxicity was observed under nutrient-rich condition.

![21 against BxPC-3](image1)

![21 against Capan-2](image2)

**Figure 5.** Survival of BxPC-3 and Capan-2 cells under nutrient-deprived medium (blue) and nutrient-rich medium (red) after 24 h incubation with compound 21. All cell viabilities are means of ± SEM, n = 3. Replicate experiments were performed and similar values were obtained. Concentrations of compound 21 investigated were 6.25, 12.5, 25, 50 and 100 \mu M. Data obtained from Zhang et al., 2016.²⁴

<table>
<thead>
<tr>
<th></th>
<th>LC_{50} (\mu M) in NDM</th>
<th>LC_{50} (\mu M) in NRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1</td>
<td>9</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Capan-2</td>
<td>5</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

**Table 3.** LC_{50} values under nutrient-deprived medium (NDM) and nutrient-rich medium (NRM) of 21 against three pancreatic cancer cell lines: PANC-1, BxPC-3, and Capan-2. Data obtained from Zhang et al., 2016.²⁴
**Medium Component Study of Compound 21**

To better understand the selective anti-proliferative activity of compound 21, that preferential cytotoxicity was observed only under nutrient-deprived medium (NDM) while no cytotoxicity under nutrient-rich medium (NRM = NDM + glucose + serum + amino acids), we initiated medium component study of compound 21 against PANC-1 cells. In this study, the effects of three essential medium components, glucose (Glu), serum (Ser), and amino acids (AA) were investigated. To better focus on the effect of serum, we also used dialyzed serum (Dia Ser). PANC-1 cells were treated with 21 under medium conditions of different combinations of these nutrient components, and the survival of cells was examined after 24h incubation by using WST-8 reagent. Medium conditions that demonstrated >25% cell death in the presence of 21 were designated “Active” and NDM-like (Figure 6A), and medium conditions that demonstrated <25% cell death were designated “Inactive” and NRM-like (Figure 6B).

In this study, the critical role of glucose on the preferential cytotoxicity of 21 was noticed. To highlight the link between observed cytotoxicity and glucose deprivation, we reported percent PANC-1 cell death at 100µM 21 under specific medium condition with reference to the glucose concentration in the medium (Figure 7). At high concentration of glucose in media, low cell death was observed after 24h incubation with 21, and at low concentration of glucose in media, high cell death was observed.
Figure 6. Survival of PANC-1 cells under different cell culture medium conditions after 24 h incubation with compound 21. All cell viabilities are means ± SEM, n = 3. Replicate experiments were performed and similar values were obtained. Concentrations of compound 21 investigated were 6.25, 12.5, 25, 50 and 100 µM. A. Media compositions that demonstrate >25% cell death in the presence of 21 are designated “Active.” B. Media compositions that demonstrate <25% cell death in the presence of 21 are designated “Inactive.” Data obtained from Zhang et al., 2016.24
Our finding showed with the presence of glucose, compound 21 is less cytotoxic; however, with the absence of glucose, 21 becomes increasingly effective, implying 21 targets a salvage pathway when glycolysis is not an option for providing energy for cancer cell survival. Autophagy\textsuperscript{13,14,27}, the regulated cytological degradation and recycling, has been found to provide a mechanism to sustain pancreatic cancer cells under nutrient starvation. This leads to our hypothesis that the mechanism of 21 is via an inhibition of autophagy.

\textbf{Figure 7.} Percent PANC-1 cell death upon exposure for 24 h to 100 μM of compound 21 under different cell culture medium conditions. All experiments were performed in triplicate, and the results are shown as means ± SEM. The final concentration of glucose (μg/mL) in each medium, plotted on a log scale, is overlayed with the corresponding viability histogram. Figure obtained from Zhang \textit{et al.}, 2016.\textsuperscript{24}
5. Introduction to Investigation

The research presented herein will be comprised of five studies with the ultimate goal to investigate the mechanism of compound 21. First, to prepare pure compound 21 for biological evaluation; second, to perform time dependent study to find out the anti-proliferative activity of 21 in respect to time; third, to compare medium component study results for three pancreatic cancer cell line, PANC-1, BxPC-3, and Capan-2; fourth, to use commercially available autophagy detection kit to learn the effect of 21 on the amount of autophagosomes; lastly, to detect the effects of 21 and autophagy inhibitors on expression of LC3-I and LC3-II with Western blotting.

1) Preparation of Compound 21

Compound 21 will be synthesized through 2 steps: deprotonation of 6-hydroxycoumarin 23 with sodium hydride in DMF, and then a Williamson ether synthesis between 6-hydroxycoumarin anion and farnesyl bromide 24 (Scheme 2).

![Scheme 2. Synthesis of farnesylated coumarin 21.](image)

2) Time Dependent Study

The aim for the time dependent study is to find out the anti-proliferative activity of compound 21 in respect to time. PANC-1 cells will be treated with and without 21 in nutrient-deprived medium and nutrient-rich medium for 8, 16, 24, 48, and 72 hours, instead of the standard 24 hours.
3) **Medium Component Study on BxPC-3 and Comparison**

(a) **New Reference Validation**

In the previous medium component study on PANC-1, cell survival of cancerous cells not treated with 21 was defined as the control or 100% cell survival for each medium condition, and cell survival of empty wells with just medium was defined as the blank or 0% cell survival. However, since we were comparing this blank with conditions where most cells died, the empty well with just medium was not a true representation of 100% cell death. Thus, this subtle difference between 100% cell death and 0% cell survival often resulted in negative cell viability values, and it was not reasonable to have more than 100% cell death.

We propose a new reference blank, the cell survival of cancerous cells treated with 1% Triton X-100 (25, Figure 8) in NDM. Triton is a widely used nonionic detergent that can kill and lyse cells at a low concentration. Such toxicity is due to the disruptive action of Triton’s polar head on the cell’s lipid bilayer, and the insertion of Triton monomer into the membrane. The exposure of cancerous cells to 1% Triton X-100 for 5 minutes could already induce 100% cell death. The survival of cancerous cells treated with Triton will serve as a better blank for the cell cytotoxicity assays.

(b) **Medium Component Study on BxPC-3**

To investigate whether there is a similar link between the preferential cytotoxicity of farnesylated coumarin 21 and glucose deprivation for other pancreatic cancer cell lines, we will
perform a medium component study on BxPC-3 cells, similarly to the one performed for PANC-1. Compound 21 will be tested in vitro for its anti-proliferative activity against BxPC-3 under eleven different medium conditions.

(c) **Comparison Among Three Pancreatic Cancer Cell Lines**

The media component studies for all three pancreatic cancer cell lines, PANC-1, BxPC-3, and Capan-2 (the data for Capan-2 were obtained by Yin Wang’16) will be compared to find whether there is a similar dependence on glucose for the preferential cytotoxicity of 21. If a similar link between selective cytotoxicity and glucose deprivation is observed for all three pancreatic cancer cell lines, this suggests 21 targets a pathway shared by most types of pancreatic cancer cells.

4) **Cyto-ID® Kit Study of Autophagy in PANC-1**

The objective of this study is to investigate whether inhibition of autophagy, the recycling of nonessential cellular components for cancer cell survival under nutrient deprivation, is a possible mechanism of compound 21 by using a commercially available Cyto-ID® Autophagy Detection kit from ENZO® Life Science Inc. The kit contains the Cyto-ID® Green Detection Reagent that could stain autophagosomes (Figure 9), the double-membrane vesicles that are formed in the process of autophagy, and emit fluorescence in the green region of the visible light spectrum. This cell analysis kit provides a rapid and specific approach for monitoring autophagic activity at the cellular level.

Starvation activates autophagy with an induction time of 1 to 4 hours, and NDM lacking all nutrients represents the condition of starvation. Rapamycin (26, Figure 9), a lipophilic macrolide antibiotic, induces autophagy by inhibiting the mammalian target of Rapamycin
(mTOR), which is a kinase that inhibits autophagy through phosphorylation of proteins involved in the process of autophagy. Rapamycin-induced autophagy results in the accumulation of autophagosomes. Chloroquine (27, Figure 9), an anti-inflammatory drug for malaria, suppresses autophagy by increasing lysosomal pH and inhibiting lysosomal activity. Chloroquine blocks the formation of autolysosome or degradation of autophagosomes, leading to the accumulation of autophagosomes as well. Both rapamycin 26 and chloroquine 27 are included as positive controls.

**Figure 9.** Schematic depiction of autophagy. Autophagosome, a double-membrane vesicle, is formed by sequestering cytosolic material. The outer membrane of the autophagosome fuses with the lysosome, and the internal material is degraded in the autolysosome. Microtubule-associated protein light chain 3 (LC3) exists in two molecular forms. LC3-I, localized in the cytoplasm, can be converted into the LC3-II, which is associated with the autophagosome membrane. Starvation and rapamycin (Rapa, 26) function as autophagy inducers, chloroquine (CQ, 27) as autophagy inhibitor by blocking degradation of autophagosomes, and wortmannin (WM, 28) as autophagy inhibitor by blocking formation of autophagosomes. Picture edited from Hashimoto et al., 2014.14
In this experiment, we will use fluorescence microscopy to visually observe the stained autophagosomes by Cyto-ID® Green Detection Reagent in PANC-1 cells treated with different medium conditions, and fluorescence microplate assay to read the fluorescence or the amount of autophagosomes in PANC-1 cells.

5) Western Blotting Analysis of Autophagy in PANC-1

One main challenge of Cyto-ID® Autophagy Detection kit is it only measures the amount of autophagosomes. However, both the induction of autophagy, by reagent like rapamycin that induces formation of autophagosomes, and the inhibition of autophagy, by reagent like chloroquine that blocks degradation of autophagosomes to autolysosomes, could result in accumulation of autophagosomes. Therefore, we will focus on Western blotting to better look into the autophagic activity in the cell.

Microtubule-associated protein 1 light chain 3 (LC3) is the protein we will use to monitor autophagy in PANC-1 cells. LC3 have two molecular forms: LC3-I localized in the cytoplasm and LC3-II bound to the autophagosome membrane (Figure 9). LC3-I converts into LC3-II by conjugating with phosphatidylethanolamine (PE), and although LC3-II has larger molecular weight than LC3-I due to the addition of PE, LC3-II still migrates faster on the gel due to its extreme hydrophobicity. LC3-I is usually detected on a gel at a molecular mass around 16kD, and LC3-II at around 14kD. The amount of LC3-II is closely related to the amount of autophagosomes and serves as a good indicator for autophagosomes formation. The concentrations of LC3-I and LC3-II will be normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping protein detected around 37kD.
Sequestosome 1 (SQSTM1/p62) is another protein we will use to detect the autophagic flux\textsuperscript{29,31}. p62 binds directly to LC3 and serves as a selective substrate of autophagy. The accumulation of p62 could suggest an inhibition of autophagy\textsuperscript{32}; however, mechanisms other than autophagy could also alter the expression of p62\textsuperscript{29}. So the concentration of p62 will be used as an additional evidence, but not as an absolute indicator.

In this experiment, the effect of compound \textbf{21} on autophagy will be compared with that of two autophagy inhibitors, chloroquine (\textbf{27}, Figure 9) and wortmannin (\textbf{28}, Figure 10). Chloroquine blocks the degradation of autophagosomes, and wortmannin, the phosphatidylinositol-3-kinase (PI3K) inhibitor, blocks the formation of autophagosomes.
RESULTS AND DISCUSSION

1) Preparation of Compound 21

The farnesylated coumarin 21 was synthesized through a Williamson ether synthesis between farnesyl bromide 24 and 6-hydroxycoumarin sodium alkoxide pre-generated from deprotonation of 6-hydroxycoumarin 23 with sodium hydride in DMF (Scheme 2). The desired product was purified through Preparative Thin Layer Chromatography (Prep TLC), and its structure confirmed by High Resolution Mass Spectrometry, Hydrogen Nuclear Magnetic Resonance (\( ^1\)H-NMR), and Carbon Nuclear Magnetic Resonance (\( ^{13}\)C-NMR).

Several small uncharacterizable peaks around 0.7-1.6 ppm in \( ^1\)H-NMR spectrum of isolated product 21 in chloroform-d were resolved with the 500MHz NMR, suggesting the presence of some possible impurities. However, after two additional purification attempts, the tiny peaks remained (Figure 10A). The \( ^1\)H-NMR spectrum of plain silica gel washed with 2% methanol in dichloromethane, the same eluting solution for purification of compound 21, also contained similar small peaks between 0.7-1.7 ppm (Figure 10B). This suggested the small peaks in the \( ^1\)H-NMR spectrum of 21 probably resulted from organic components in TLC silica gel that were dissolved by the eluting solution during the process of purification.
Figure 10. $^1$H-NMR spectra of compound 21 purified through Prep TLC (A) and plain silica gel washed with 2% methanol in dichloromethane (B) in chloroform-d.
2) Time Dependent Study

The time dependent study aimed to find out the anti-proliferative activity of compound 21 in respect to time. PANC-1 cells were treated with and without 21 in nutrient-deprived medium (NDM) and nutrient-rich medium (NRM) for 8, 16, 24, 48, and 72 hours (Figure 11). Our previous studies have found that 21 exhibits selective cytotoxicity against PANC-1 cells under nutrient-deprived conditions with an LC$_{50}$ value of 9µM for 24-hour incubation (Table 3). In this study, exposure of PANC-1 cells to 10µM 21 for 24 hours induced 50% cell death in NDM (Figure 11A). Furthermore, the presence of 21 began to show anti-proliferative activities after 8 hours of exposure in NDM, and PANC-1 cells reached 100% cell death in NDM, both with and without 21, after 48 hours of incubation.

**Figure 11.** Survival of PANC-1 cells under nutrient-deprived medium (blue) and nutrient-rich medium (red), and PANC-1 cells treated with 10µM compound 21 under nutrient-deprived medium (green) and nutrient-rich medium (orange) after 8-, 16-, 24-, 48-, and 72-hour incubations. The survival of PANC-1 not treated with compound 21 under nutrient-rich conditions after 8h incubation was defined as 100% survival reference. Graph B had media changed at 24h and 48h incubation. All cell survivals are means of ±SEM, n = 3. Replicate experiments were performed and similar values were obtained.
At the same time, the presence of $21$ didn’t exhibit any anti-proliferative effect against PANC-1 in NRM until 72 hours of incubation (Figure 11A). The survival of PANC-1 cells under nutrient-rich conditions for 8-hour incubation was defined as 100% survival reference. The increase of cell survival represented continued cell growth in NRM, until 72 hours of incubation, when there was a sudden drop in of cell survival, and more pronounced drop with the presence of $21$. It was hypothesized that the drop could have been caused by the depletion of nutrients in NRM after 48 hours of incubation, so that the NRM became NDM like, resulting in cell inhibition in the depleted medium and more severe cell death with the exposure to $21$.

To test this hypothesis, a similar experiment was conducted where the media were changed after 24-hour and 48-hour incubations (Figure 11B). Therefore, PANC-1 cells were provided with sufficient nutrients in NRM for the duration of the experiment. As expected, there was no more survival drop after 72 hours of incubation, but a steady increase of cell growth, and no anti-proliferative activity of $21$ was observed under nutrient-rich conditions. With even longer incubation, PANC-1 cell growth may reach an equilibrium because of the carrying capacity.
3) Medium Component Study on BxPC-3 and Comparison

(a) New Reference Validation

To better represent 100% cell death, we decided to use the cell survival of pancreatic cancer cells treated with 1% Triton X-100 (25, Figure 8) in NDM as reference, instead of the previous cell survival of empty well with just medium as blank. To validate the credibility of this new reference, we repeated the same media component study on PANC-1 with this new blank (Figure 8). In Figure 12, the dashed lines represent the cell viabilities with the old blank, and the solid lines represent the cell viabilities with the new blank. The new blank helped to eliminate the problem of negative cell survival. Because of the biological variability, there are some fluctuations between the data generated using old blank and those with the new blank, but the overall trends were consistent.
Figure 12. Comparison of the viabilities of PANC-1 cells under different medium conditions after 24-hour incubation with compound 21 with old blank (just media, dashed line) and with new blank (cells treated with 1% Triton X-100, solid line). All cell survivals are means ± SEM, n = 3. Replicate experiments were performed and similar values were obtained. Concentrations of compound 21 investigated were 6.25, 12.5, 25, 50 and 100 µM.
(b) *Medium Component Study on BxPC-3*

To investigate whether there is a similar link between the preferential cytotoxicity of compound 21 and glucose deprivation for other pancreatic cancer cell lines, we performed a medium component study on BxPC-3 cells, similarly to the one performed for PANC-1. Compound 21 was tested *in vitro* for its anti-proliferative activity against BxPC-3 under different medium conditions (Figure 13). Nutrient-rich medium (NRM) containing all three essential medium components: amino acid (AA), glucose (Glu), and serum (Ser), and nutrient-deprived medium (NDM) lacking all three nutrients were selected as references. We used dialyzed serum (Dia Ser) with glucose concentration of less than 5µg/ml, to minimize the effect of glucose in serum with glucose concentration of 96µg/ml. A total of 11 medium conditions were tested, and the survival of BxPC-3 cells was examined after 24 hours of incubation with 21 by the WST-8 cell counting reagent.

Cell survival of BxPC-3 cells not treated with 21 was defined as 100% cell survival for each medium condition, and cell survival of BxPC-3 treated with 1% Triton X-100 defined as 0% cell survival. Media conditions that demonstrate >35% cell death in the presence of 21 are considered “Active” or NDM-like (Figure 13A), and media conditions that demonstrate <35% cell death in the presence of 21 are considered “Inactive” or NRM-like (Figure 13B). The medium combination of NDM and dialyzed serum and/or amino acid still exhibited a similar effect on the cytotoxicity of 21 as NDM (Figure 13A), indicating the relatively unimportant role of Dia Ser and AA in the preferential cytotoxicity of 21. On the other hand, the presence of glucose or low levels of glucose in serum rendered the medium inactive and behave like NRM (Figure 13B). The result showed a similar link between observed cytotoxicity and glucose deprivation in media.
Figure 13. Survival of BxPC-3 cells under different cell culture medium conditions after 24-hour incubation with compound 21. All cell survivals are means ± SEM, n = 3. Replicate experiments were performed and similar values were obtained. Concentrations of compound 21 investigated were 6.25, 12.5, 25, 50 and 100 μM. A. Media compositions that demonstrate >35% cell death in the presence of 21 are designated “Active.” B. Media compositions that demonstrate <35% cell death in the presence of 21 are designated “Inactive.”
(c) Comparison Among Three Pancreatic Cancer Cell Lines

Figure 14 compiles the results of the media component studies for all three pancreatic cancer cell lines, PANC-1, BxPC-3, and Capan-2 (the data for Capan-2 were obtained by Yin Wang’16). The cell death (Figure 14A) and cell survival (Figure 14B) of cancerous cells treated with 100 μM compound 21 for 24 hours in various cell culture media conditions are reported, as well as the final glucose concentration on a log scale in each condition.

A similar overall trend is observed for all three pancreatic cancer cell lines: higher glucose concentration in medium results in lower cell death or higher cell survival when treated with 21, in other words, less anti-proliferative activity of 21. In general, the similar trend indicates the same link between the selective cytotoxicity of 21 and glucose deprivation for all these three cell lines, suggesting 21 targets the pathway shared by most types of pancreatic cancer cell lines.

It is also important to point out that Capan-2, compared with PANC-1 and BxPC-3, is much more sensitive to any glucose concentration in medium. The high sensitivity is highlighted by the medium conditions of NDM+Dia Ser and NDM+Dia Ser+AA, in which the final glucose concentration is only 5μg/mL, behaving as NDM-like for PANC-1 and BxPC-3, but NRM-like for Capan-2. This difference in sensitivity might result from the intrinsic differences in cell characteristics for these three cell lines.
Figure 14. Percent PANC-1 (green), BxPC-3 (blue), and Capan-2 (orange, data generated by Yin Wang ’16) cell death (A) and cell survival (B) upon exposure for 24 hours to 100 µM of compound 21 in cell culture media conditions of various nutrient components (glucose, amino acid, serum, and dialyzed serum). 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.
4) **Cyto-ID® Kit Study of Autophagy in PANC-1**

The objective of this study is to investigate whether inhibition of autophagy, the recycling of nonessential cellular components, is a possible mechanism of compound 21 by looking into the amount of autophagosomes with a commercially available Cyto-ID® Autophagy Detection kit from ENZO® Life Science Inc. Rapamycin (26, Figure 9), autophagy inducers, and chloroquine (27, Figure 9), autophagy inhibitor by blocking formation of autolysosome, will lead to accumulation of autophagosomes and are used as positive controls in this experiment.

**(a) Fluorescence Microscopy**

The first series of experiments with this kit was using fluorescence microscopy to visually observe the stained autophagosomes. To determine the optimal condition of positive control, PANC-1 cells were treated with various combinations of different concentrations of chloroquine 27 (10, 50, and 100µM) and rapamycin 26 (0.5, 1, and 2µM) under both NRM and NDM for 18 hours, incubated with Cyto-ID® Green Detection Reagent for 30 minutes while protected from light, and analyzed with fluorescence microscopy by using FITC filter set (Figure 15). The combination of 50µM chloroquine and 1µM rapamycin gave the most intense signal, and was defined as the positive control.

![Figure 15. Fluorescence image of PANC-1 cells stained with Cyto-ID®. PANC-1 cells were plated in a 8-well chamber plate overnight, treated with different combinations of chloroquine (10, 50, and 100µM) and rapamycin (0.5, 1, and 2µM) concentrations under both NRM and NDM for 18 hours, and incubated with Cyto-ID® Green Detection Reagent for 30 minutes while protected from light. The pictures were taken with fluorescence microscopy with FITC filter set. The green dots are the autophagosomes.](image-url)
The next goal was to determine the effect of compound 21 on the autophagic activity of cells. PANC-1 cells were treated without 21, with 10µM 21, with positive control, and with the combination of positive control and 21 under both NRM and NDM (Figure 16). In this experiment, there was a slight increase in fluorescence from cells in NDM compared to those in NRM, indicating the effect of starvation on inducing autophagy. The cells also demonstrated intense fluorescence when treated with positive control, but barely any fluorescence when treated with just media or media and 21. Therefore, no valid conclusion could be reached about differences in fluorescence of PANC-1 untreated and treated with 21.

![Figure 16. Fluorescence image of PANC-1 cells stained with Cyto-ID®. PANC-1 cells were plated in a 8-well chamber plate overnight, treated with plain media, with 10µM 21, with positive control (50µM chloroquine and 1µM rapamycin), and with the combination of 21 and positive control under both NRM and NDM for 20 hours, and incubated with Cyto-ID® Green Detection Reagent for 30 minutes while protected from light. The pictures were taken with fluorescence microscopy with FITC filter set. The green dots are the autophagosomes.](image)

The rapid fluorescence decay of Cyto-ID® Green Detection Reagent within the range of minutes was a significant challenge working with this kit. Even with the best efforts to prevent any light exposure, there was always a delay in time from when light hit the cell and when picture was taken manually. In addition, when light hit one well, the cells in other wells would inevitably receive some scattered light, decreasing the fluorescence signal. As result, the
fluorescence images could not serve as conclusive evidence for the effect of 21 on autophagosomes. Consequently, we decided to move to a fluorescence microplate assay, which could not only minimize the effect of fluorescence decay when reading with an automated microplate reader, but also provide with quantitative data.
(b) Fluorescence Microplate

In the first attempt with the microplate assay, PANC-1 cells were treated under the same eight conditions as the previous fluorescence microscopy experiment for 24 hours, and the blank employed was assay buffer provided in the kit (Figure 17). The fluorescence readings for cells treated with positive control were high, and even higher when 21 was present for both NRM and NDM, indicating possible effect of 21 on autophagic activity. However, the differences between cells untreated and treated with 21 for both NRM and NDM were not significant.

![Fluorescence Microplate Assay — 24h incubation](image)

**Figure 17.** Fluorescence reading of PANC-1 cells stained with Cyto-ID®. PANC-1 cells were plated in a 96-well clear bottom black microplate overnight, treated with plain media, with 10µM 21, with positive control (50µM chloroquine and 1µM rapamycin), and with the combination of 21 and positive control under both NRM and NDM for 24 hours, and incubated with Cyto-ID® Green Detection Reagent for 30 minutes while protected from light. The plate was top read with a fluorescence microplate reader with excitation at 480nm and emission at 530nm. Three replicate wells for each media condition, nine readings for each well for each read, and three reads were employed. Readings below the reading of background, which was assay buffer, were removed. The reported fluorescence is the mean of readings subtracted by the background reading, and the error bar represents SEM.
A few problems arose when analyzing the fluorescence data. The first was a high background reading. The reported fluorescence was calculated by subtracting the background reading from the mean of readings for the cells. The background was assay buffer without any cells, which should not have any fluorescence, but gave an average reading of 4.4, while the readings for the cells treated with Cyto-ID® reagent were around 4.7-5.3, and subtracting the background from the actual reading resulted in small reported fluorescence output of around 0.3-0.9. Another problem was that many readings were below the background reading, indicating these readings didn’t hit the cells. After consulting with the technical support from ENZO®, we improved the experimental design. To solve the problem of high background reading, the blank would be changed to cells treated with the same medium condition but without Cyto-ID® reagent, since the fluorescence readings for cells and for just media were not comparable. This new blank could also eliminate the interference of the possible fluorescence of compound 21. To reduce readings below background reading, the incubation time was shortened, so that fewer cells would die and detach during incubation, especially under nutrient-deprived conditions. In addition, 24 hours was too long to observe the effect of starvation. Lastly, the microplate would be read from the bottom instead of from the top, so that the cells could be brought as close as possible to the detector and the noise from overlying media could be avoided.

With these modifications to the experimental design in place, two more attempts of 4 and 8 hours incubation (Figure 18A) and 2 hours incubation (Figure 18B) with PANC-1 cells under the same eight media conditions were performed.
Fluorescence Microplate Assay — 4h and 8h incubation

Figure 18. Fluorescence reading of PANC-1 cells stained with Cyto-ID®. PANC-1 cells were plated in a 96-well clear bottom black microplate overnight, treated with plain media, with 10µM chloroquine and 1µM rapamycin, and with the combination of positive control and 21 under both NRM and NDM for 4 and 8 hours (A) and 2 hours (B), and incubated with Cyto-ID® Green Detection Reagent for 30 minutes while protected from light. The plate was bottom read with a fluorescence microplate reader with excitation at 480nm and emission at 530nm. Three replicate wells for each media condition, nine readings for each well for each read, and two reads were employed. Readings below the reading of background, which was cells treated with the same conditions but without Cyto-ID®, were removed. The reported fluorescence is the mean of readings subtracted by the background reading, and the error bar represents SEM.
Starvation has an induction time of 1-4 hours. With the 2-hour incubation (Figure 18B), there was a slight increase of fluorescence in NDM compared to that in NRM, which might suggest the effect of starvation, yet the difference was very small. In NRM, the presence of compound 21 resulted in an increase of fluorescence, representing higher level of autophagosomes. The increase was evident when comparing the readings for NRM 2h/4h and those for NRM+21 2h/4h, and the reading for NRM+positive control and that for NRM+21+positive control. One possible explanation for this increase of autophagosomes by 21 is under nutrient-rich conditions, glycolysis is available to provide energy for PANC-1, and the presence of 21 triggers PANC-1 cells to initiate autophagy as a cytoprotective mechanism, resulting in the accumulation of autophagosomes, so that cells could still survive with presence of 21 under NRM.

In NDM, on the other hand, the presence of 21 did not have much effect on fluorescence. A possible hypothesis could be that under nutrient-deprived conditions, glycolysis is already not available to provide energy, the presence of 21 triggers cells to initiate autophagy as cytoprotective mechanism and as energy source, but autophagy is inhibited by 21 in NDM, so the autophagosomes remain unaffected at baseline level and cells die with presence of 21 under NDM. Based on the data obtained, there was no conclusive evidence to support either hypothesis. Nevertheless, 21 did seem to have some effect on the level of autophagosomes in NRM. A repeated assay is needed to validate the finding, but unfortunately, the Cyto-ID® reagent was used up and the kit was too expansive to experiment with.
5) Western Blotting Analysis of Autophagy in PANC-1

(a) Effects of 21 and Autophagy Inhibitors on Conversion of LC3-I to II

In this experiment, cell lysates were prepared by treating PANC-1 cells with plain media, 10µM 21, 25µM chloroquine (27, Figure 9), or 1µM wortmannin (28, Figure 9) under both nutrient-deprived medium (NDM) and nutrient-rich medium (NRM) for 24 hours. Due to the short half-life of wortmannin14, PANC-1 was incubated with wortmannin for 4 hours, and then in either NRM or NDM for the other 20 hours. The protein concentrations of cell lysate supernatants were measured with NanoDrop. Supernatants of equal amounts of protein were mixed with Laemmli sample buffer, boiled for 5 minutes, and ran on a 4-20% Mini-PROTEAN® TGX gel. The proteins were then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane, which was blocked with blocking buffer, then treated with polyclonal LC3, GAPDH, and p62 antibodies separately, and then secondary antibody. The ladder used was WesterC Standards detected by Strep Tactin-HRP, and the band densities were measured with Image Lab (Figure 19).

Chloroquine is an autophagy inhibitor by blocking the degradation of autophagosomes, and LC3-II is the form of LC3 that is attached to the membrane of autophagosomes. Western blotting showed that treating PANC-1 cells with chloroquine under both NRM and NDM results in an accumulation of LC3-II (Figure 19). On the other hand, wortmannin is an autophagy inhibitor by blocking the formation of autophagosomes, and as shown by the Western blotting, treating PANC-1 cells with wortmannin under both NDM and NRM decreases the level of LC3-II. PANC-1 cells under nutrient-deprived conditions in general also showed an increase of conversion of LC3-I to LC3-II than those under nutrient-rich conditions, suggesting the induction of autophagy by starvation.
Figure 19. Effects of 21 and autophagy inhibitors on expressions 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, 10µM 21, 25µM chloroquine (CQ), or 1µM wortmannin (WM) under both nutrient-deprived medium (NDM) and nutrient-rich medium (NRM) for 24 hours. Cell lysate supernatants with Laemmli sample buffer were loaded and ran on a 4-20% Mini-PROTEAN® TGX gel. Proteins were transferred from gel to PVDF membrane, which was blocked with blocking buffer, then treated with polyclonal LC3, GAPDH, and p62 antibodies separately, and secondary antibody. The ladder on the rightmost lane was WesterC Standards detected by Strep Tactin-HRP. The band densities were measured with Image Lab and reported in the table.
Expressions of LC3-I and LC3-II in PANC-1 did not have significant changes with the 24-hour treatment of compound $21$ under NRM (Figure 19), agreeing with what we previously knew about compound $21$ that it shows no cytotoxicity under NRM. On the other hand, treating PANC-1 cells with $21$ under NDM for 24 hours increased the conversion of LC3-I to LC3-II (the LC3-II to LC3-I ratio is 1.57 under NDM and 1.88 under NDM+$21$), possibly suggesting an inhibition of autophagy in the same way as chloroquine inhibiting autophagy. This potential inhibition of autophagy under nutrient-deprived conditions agrees with the preferential cytotoxicity of $21$ only under glucose deprivation.

p62, or SQSTM1, is another protein we monitored. The accumulation of p62 could suggest an autophagy inhibition$^{32}$. However, treating PANC-1 cells with autophagy inhibitors, chloroquine and wortmannin, under both NRM and NDM resulted in a decrease in the level of p62, suggesting the levels of p62 were influenced by pathways other than autophagy in the cells. Therefore, the expression of p62 was not used to detect the effect of $21$ on the autophagic flux in PANC-1 cells.
(b) Conversion of LC3-I to II Dependent on Time of Incubation

We have noticed that the structure of 21 shares some similarity with that of chloroquine, the autophagy inhibitor (27, Figure 9). To better investigate the effect of 21 on the autophagic activity in PANC-1 and to compare that with the effect of chloroquine, we closely looked into the survival of PANC-1 cells and expressions of LC3-I and LC3-II as dependent on time of treatment with 21 or chloroquine. In this study, PANC-1 cells were treated with plain medium, 10µM 21, or 25µM chloroquine under both nutrient-deprived medium (NRM) and nutrient-rich medium (NDM) for 3, 7, 12, and 24 hours. Cytotoxicity assays were performed to find out the cell survival (Figure 20A), and Western blotting analysis were performed on cell lysates treated under the same conditions to detect the expressions of LC3-I and LC3-II (Figure 20B).

Under nutrient-rich conditions, both 21 and chloroquine didn’t exhibit any cell cytotoxicity against PANC-1 (Figure 20A). In addition, the levels of LC3 proteins didn’t change significantly with treatment of 21 in NRM over different times of incubation; however, treating PANC-1 cells with chloroquine in NRM led to an accumulation of LC3-II with longer time of incubation, suggesting autophagy is inhibited by chloroquine even under NRM.

Under nutrient-deprived conditions, anti-proliferative activities of 21 and chloroquine were not observed for the first 7 hours of incubation, probably because PANC-1 cells still had enough leftover nutrients when media were changed to NDM, so that they didn’t have to trigger alternate metabolic pathways for survival for the initial few hours of incubation. Both treatments of 21 and chloroquine under NDM exhibited anti-proliferative activities after 24 hours of incubation, and led to accumulations of LC3-II over time, suggesting 21 behaves as chloroquine in inhibiting autophagy under nutrient-deprived conditions.
Figure 20. **A.** Survival of PANC-1 cells treated with plain medium, 10μM 21, or 25μM chloroquine (CQ) under nutrient-deprived medium (NDM) and nutrient-rich medium (NRM) for 3, 7, 12, and 24 hours. The survival of PANC-1 after 0 hour incubation was defined as 100% survival. All cell survivals are means of ±SEM, n = 3. Replicate experiments were performed and similar values were obtained. **B.** Western blot analysis was performed on cell lysates after treating PANC-1 cells with plain media, 10μM 21, or 25μM chloroquine under both NDM and NRM for 3, 7, 12, and 24 hours. Cell lysate supernatants with Laemmli sample buffer were loaded and ran on 4-20% Mini-PROTEAN® TGX gels. Proteins were transferred from gels to PVDF membranes, which were blocked with blocking buffer, treated with polyclonal LC3 and GAPDH antibodies separately, and secondary antibody.
CONCLUSION

The link between preferential cytotoxicity of compound 21 against PANC-1 and glucose deprivation suggested 21 targets a pathway when glycolysis is not an option for cancer cell survival. In this research, the anti-proliferative mechanism of 21 was investigated. The small peaks in 1H-NMR spectrum of 21 had been identified to be the organic components in TLC silica gel that were dissolved in the process of purification. In the time dependent study, 21 exhibited anti-proliferative activities from 8 hours to 48 hours of exposure in NDM, suggesting its mechanism of action has a relatively short induction time. In the medium component study, a similar link between selective cytotoxicity and glucose deprivation was observed for all three pancreatic cancer cell lines, PANC-1, BxPC-3, and Capan-2, indicating 21 targets a pathway shared by most types of pancreatic cancer cells.

In the Cyto-ID® Autophagy Detection kit study, autophagosomes were visually observed with a commercially available fluorescence stain. Although 21 did not have much effect on fluorescence or the amount of autophagosomes under NDM, an increase of fluorescence was observed by the presence of 21 under NRM, suggesting the possible involvement of 21 in the process of autophagy. In the Western blotting analysis of autophagy, 21 was found to have a similar effect as chloroquine, an autophagy inhibitor, on increasing the conversion of LC3-I to LC3-II in PANC-1, resulting in an accumulation of LC3-II. Furthermore, while 21 and chloroquine exhibited anti-proliferative activity only under NDM, 21 increased conversion to LC3-II over time only under NDM, but chloroquine increased conversion to LC3-II under both NRM and NDM. This finding suggests while chloroquine inhibits autophagy under all conditions, 21 inhibits autophagy only under NDM. Understanding its anti-proliferative mechanism is one step toward the potential use of 21 as novel therapy against pancreatic cancer.
METHODS AND MATERIALS

Preparation of Compound 21

Synthesis of 6-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)-2H-chromen-2-one

Compound 21 was synthesized as described by Scheme 2\textsuperscript{26}. All reagents and solvents were purchased commercially from Sigma-Aldrich\textsuperscript{®}. To an oven-dried 100 mL round bottom flask prepared with a magnetic stirring bar, a rubber septum cover, and a nitrogen inlet, 6-hydroxycoumarin (1.14 g, 7.0 mmol) and 40 mL of anhydrous N,N-dimethylformamide (DMF) were added. The solution was cooled to 0 °C in a salt-ice bath and sodium hydride (280 mg of 60% mineral oil suspension, 7.0 mmol) was added to the flask. The solution was then stirred at 0 °C for 30 minutes. Farnesyl bromide (4.5 mL, 16.6 mmol) dissolved in 10 mL of anhydrous DMF was cooled to 0°C and added dropwise to the reaction flask through the rubber septum using a syringe. The reaction mixture was left stirring under nitrogen with warming to room temperature overnight and concentrated in vacuum.

Purification of Compound 21

The desired product was purified via Preparative Thin Layer Chromatography (7:3 hexane:ethyl acetate). The eluting solution was 2% methanol in dichloromethane.

Characterizations of Compound 21

The structure of 21 was confirmed by High Resolution Mass Spectrometry, Hydrogen Nuclear Magnetic Resonance (\textsuperscript{1}H-NMR), and Carbon Nuclear Magnetic Resonance (\textsuperscript{13}C-NMR). HRMS was performed on a 70-VSE mass spectrometer at the University of Illinois at Urbana-Champaign Mass Spectrometry Facility. 21 was diluted in chloroform-d, and \textsuperscript{1}H- and \textsuperscript{13}C-NMR spectra were recorded on a Bruker Ascend\textsuperscript{TM} 500 spectrometer at 500 MHz (\textsuperscript{1}H-NMR) or 125 MHz (\textsuperscript{13}C-NMR). Chemical shifts (δ) were reported in parts per million (ppm) relative to
tetramethylsilane (TMS), and coupling constants were reported in Hz. $^1$H-NMR $\delta$ 1.60 -1.76 (12H), 1.96-2.12 (8H), 4.57 (d, 2H, J=6 Hz), 5.10 (m, 2H), 5.48 (t, 1H), 6.43 (d, 1H, J=9 Hz), 6.92-7.66 (4H); $^{13}$C-NMR $\delta$ 16.04, 16.74, 17.70, 25.70, 26.17, 26.71, 29.70, 39.53, 39.69, 111.08, 117.02, 117.83, 118.93, 119.15, 129.13, 123.53, 124.27, 131.38, 135.57, 141.94, 143.25, 148.42, 155.35, 161.04. HRMS (CI) Calcd. for C$_{24}$H$_{31}$O$_{3}$: 367.22733; found: 367.22801.

Tumor Cell Culture and Media Preparations

Cell Lines

Human pancreatic cancer cell lines, PANC-1, BxPC-3, and Capan-2 were obtained from American Type Culture Collection (ATCC).

Tumor Cell Culture Procedures

To a cell-containing T-75/T-25 culture flask, a 2-5 mL aliquot of filtered trypsin-EDTA was added to detach the cells after the aspiration of DMEM and a PBS wash. The trypsin-containing flask was placed in the incubator (37°C and 5% CO$_2$) for 3 minutes. Detached cells were transferred to a 15 mL centrifuge tube and pelleted for 3 minutes at 1000 rpm. The supernatant of the resulting content was aspirated. The cell pellet was re-suspended thoroughly in the complete DMEM and transferred to a new T-75/T-25 flask with additional DMEM to reach a final volume of 25/5 mL, and store in the incubator (Sheldon Manufacturing Inc.)

Preparation of Dulbecco Modified Eagle’s Medium (DMEM)

Pancreatic cancer cells were cultured in Dulbecco Modified Eagle’s Medium (DMEM). To prepare DMEM, one bottle of DMEM powder (Sigma-Aldrich) was dissolved in 800 mL of HPLC grade water. Sodium bicarbonate solution (49.1 mL) and antibiotic-antifungal solution (10 mL, 100x) were added to the mixture. The volume was brought to 900 mL and the solution was
adjusted to a pH between 7-7.4. Heat-inactivated fetal bovine serum (FBS, 100 mL) was added to the neutralized solution and filtered via a 0.2-μm Corning filter. The medium was stored at 4 °C. Cells were passaged once to twice a week by trypsinization.

**Preparation of Nutrient-Deprived Medium (NDM), Nutrient-Rich Medium (NRM) and Special Media Conditions**

For all medium conditions, the following electrolytes and vitamin solution were added in concentrations as follows: CaCl$_2$(2H$_2$O), 265 mg/L; Fe(NO$_3$)(9H$_2$O), 0.1 mg/L; KCl, 400 mg/L; MgSO$_4$(7H$_2$O), 200 mg/L; NaCl, 6400 mg/L; NaHCO$_3$, 700 mg/L; NaH$_2$PO$_4$, 125 mg/L; phenol red, 15 mg/L; HEPES buffer (25mM, pH 7.4); and MEM vitamin solution (1x/L) (Life Technologies, Inc., Rockville, MD), which completed the preparation of the NDM. For NRM, additional nutrients were supplement at concentrations as follows: D-glucose, 1000mg/L; L-glutamine, 2 mM; MEM amino acids solution and MEM nonessential amino acids solution (Life Technologies, Inc.), 20 mL and 10 mL respectively for 1 L medium; FBS, 100 mL for 1 L medium. For all special media combinations, corresponding combinations of nutrients were added at concentrations identical to those in NRM. For media with dialyzed serum, the same concentration of dialyzed serum (Life Technologies, Inc.) was added as a replacement of FBS (100mL for 1 L medium).

**Cytotoxicity Assays**

**General Procedures**

*In vitro* cytotoxicity assay: Pancreatic cancer cells (PANC-1, BxPC-3, or Capan-2), were seeded in 96-well plates at a density of 23,000 cells per well and incubated in DMEM, at 37 °C, 5% CO$_2$ for 24 h. After rinsing with PBS, cells were subjected to the addition of NRM, NDM, or special media conditions. Serially diluted solutions of compounds 21 (5.5% v/v DMSO in NDM)
were added to the cells up to a series of concentrations of 100 μM, 50 μM, 25 μM, 12.5 μM and 6.25 μM, followed by a 24 h incubation at 37 °C, 5% CO$_2$. Cell morphology was monitored under an inverted microscope. After washing with PBS, cytotoxicity was assessed by the addition of DMEM containing 10% WST-8 cell counting reagent (Dojindo Molecular Technologies). Following a 3 h incubation at 37 °C, 5% CO$_2$, absorbance values were measured with a plate reader (SpectraMax® M3 from Molecular Devices) at 450 nm, and cell viability was calculated using the equation:

\[
\%_{\text{cell viability}} = \frac{\text{Abs}_{\text{test}} - \text{Abs}_{0\%}}{\text{Abs}_{100\%} - \text{Abs}_{0\%}} \times 100\%
\]

**Time Dependent Study**

Instead of variable compound concentrations and constant 24 h incubation, PANC-1 cells were treated with and without 10μM 21 in NDM and NRM for 8, 16, 24, 48, and 72 hours. The absorbance of PANC-1 not treated with compound 21 in NRM after 8h incubation was defined as 100% viability reference, and the absorbance of plain DMEM was defined as 0% viability reference. Two replicate experiments were conducted, and similar results were obtained.

**Medium Component Study**

A total of 11 assays for 11 different medium conditions were prepared following the general cytotoxicity assay procedures. The absorbance of cancerous cells (PANC-1 or BxPC-3) not treated with 21 was defined 100% viability reference for each medium condition, and the absorbance of cancerous cells treated with 1% Triton X-100 (Sigma) was defined as 0% viability reference. At least three replicate experiments were conducted for each medium condition, and similar results were obtained.
**Autophagy Time Dependent Study**

Instead of variable compound concentrations and constant 24 h incubation, PANC-1 cells were treated with plain medium, 10µM 21, or 25µM chloroquine in NDM and NRM for 3, 7, 12, and 24 hours. The absorbance of PANC-1 after 0 hour incubation was defined as 100% viability reference, and the absorbance of PANC-1 treated with 1% Triton X-100 was defined as 0% viability reference. Three replicate experiments were conducted, and similar results were obtained.

**CYTO-ID Autophagy Detection Kit**

**Materials**

Cyto-ID® Autophagy Detection kit was purchased from ENZO® Life Science Inc. The kit included Cyto-ID® Green Detection Reagent, rapamycin, chloroquine, and assay buffer.

**Fluorescence Microscopy**

PANC-1 cells were seeded in a 8-well chamber plates at a density of 1000-5000 cells per well and incubated in DMEM, at 37 °C, 5% CO$_2$ for 24 h. After rinsing with PBS, cells were subjected to the addition of NRM or NDM. To set the positive reference, PANC-1 cells were treated with different combinations of chloroquine (10, 50, and 100µM) and rapamycin (0.5, 1, and 2µM) for 18 hours at 37 °C, 5% CO$_2$. To test the effect of 21 on autophagy, PANC-1 cells were treated with 10µM 21, 50µM chloroquine and 1µM rapamycin as positive control, and combination of 21 and positive control for 20 hours at 37 °C, 5% CO$_2$. After washing with assay buffer twice, cells were incubated with Cyto-ID® Green Detection Reagent (2 µL for 1 mL assay buffer) for 30 minutes while protected from light at 37 °C, 5% CO$_2$. After washing with assay buffer twice, the plastic chamber was removed. Mounting media was added one dot per well, and
coverslip was overlaid. The fluorescence images were taken by Nikon 80i Fluorescent Microscope with a standard FITC filter set.

Fluorescence Microplate

PANC-1 cells were seeded in a 96-well clear bottom black microplate at a density of 60,000 cells per well and incubated in DMEM, at 37 °C, 5% CO₂ for 24 h. After rinsing with PBS, cells were treated with plain medium, 10µM 21, 50µM chloroquine and 1µM rapamycin as positive control, and combination of 21 and positive control in NRM or NDM for desired incubation time at 37 °C, 5% CO₂. After washing with assay buffer twice, cells were incubated with Cyto-ID® Green Detection Reagent (1 µL for 1 mL assay buffer) for 30 minutes while protected from light at 37 °C, 5% CO₂. After washing with assay buffer twice, cells were added with fresh assay buffer, and fluorescence values were bottom read with a fluorescence microplate reader (SpectraMax® M3 from Molecular Devices) with excitation at 480nm and emission at 530nm. Fluorescence readings below the reading of background, which was cells treated with the same conditions but without Cyto-ID®, were removed. The reported fluorescence is the mean of readings subtracted by the background reading.

Western Blotting Analysis of Autophagy

Preparation of Cell Lysate

PANC-1 cells were seeded in T-75 flasks or 6-well plates at a density of 600,000 cells per well, and incubated in DMEM, at 37 °C, 5% CO₂ for 24 h. After rinsing with PBS, cells were treated with plain medium, 10µM 21, 25µM chloroquine (Sigma), or 1µM wortmannin (Sigma) in NRM or NDM for 24 hours or desired incubation time at 37 °C, 5% CO₂. Due to the short half-life of wortmannin, PANC-1 was incubated with wortmannin for 4 hours, then the media
was removed, and cells incubated in fresh NRM or NDM for the other 20 hours. Lysing solution was freshly made by diluting 100X Halt™ Protease Inhibitor Cocktail (Thermo Scientific) in cold Pierce RIPA Buffer (Thermo Scientific). After washing with cold PBS twice, cold lysing buffer was added 1 mL for each T-75 flask or 150 µL per well for 6-well plate, and kept on ice for 5 min with occasion stirring for uniform spreading of lysing buffer. Each cell lysate was collect with a cell scraper and transferred into a micro-centrifuge tube, and centrifuged with Eppendorf Centrifuge 5804R at 14000 rpm for 15 min at 4 ºC. The protein concentrations of cell lysate supernatants were measured with NanoDrop 2000 Spectrophotometer (Thermo Scientific). Cell lysates were stored in -20 ºC in aliquots.

**Western Blotting**

Cell lysate supernatants containing equal amounts of protein were subjected to 4-20% or any kD Mini-PROTEAN® TGX gels (Bio-Rad), after addition of Laemmli sample buffer (Bio-Rad) and boiling for 5 min. 5 µL Precision Plus Protein WesternC Standards (Bio-Rad) or 7 µL Precision Plus Protein Dual Color Standards (Bio-Rad) was loaded for each gel. The running buffer for the TGX gels were 1X 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. Gel was opened carefully, and the “sandwich” was assembled as below:
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, then blocked in blotting buffer for 60 min at room temperature. Washing buffer was prepared by adding 50 mL 20X Tris-buffered saline (dissolving 193.6 g Tris base and 640 g NaCl in 4 L deionized H₂O, and adjusting pH to 7.6), 5 mL 20% Tween20 (American Bioanalytical), and bring the volume to 1 L with deionized water. Blotting buffer was prepared by dissolving 2.5 g blocking reagent (Bio-Rad) in 50 mL washing buffer. The membrane was then incubated on shaker with polyclonal LC3B/MAP1LC3B Antibody (1:1000, Novus) for 4 hours, and GAPDH Antibody (1:3000, Rockland) for 1-2 hours, and optionally polyclonal p62/SQSTM1 Antibody (1:1000, Novus) for 2-3 hours, with a quick wash between each primary antibody. After a thorough wash, membrane was incubated with Goat anti-Rabbit IgG (1:5000, Thermo) for 1h, and optionally StrepTactin-HRP Conjugate (1:10000, Bio-Rad) for 2-4 hours. After another thorough wash, membrane was sprayed with Chemiluminescent HRP Antibody Detection Reagent (Danville Scientific Inc.) and left for 1 min. The blot was placed between two transparent plastic sheets, and was visualized with ChemiDoc™ MP Imaging System (Bio-Rad). The density of each band of LC3-II was measured using Image Lab software. Densities were described in relation to the densities of GAPDH for each medium condition.
REFERENCES

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$^{13}$C-NMR of compound 21 in chloroform-d
Replicate cytotoxicity assays for the time dependent study

Without Media Change (RZ 12.05.15-1)

Cell Survival (%) vs Time (h)

With Media Change (RZ 12.05.15-2)

Cell Survival (%) vs Time (h)
Compiled replicate cytotoxicity assays for the medium component study with PANC-1

**PANC-1 in NRM**

![Graph showing cell viability (%)](image)

**PANC-1 in NDM**

![Graph showing cell viability (%)](image)
PANC-1 in NDM + Ser

Cell Viability (%)

Concentration (µM)

PANC-1 in NDM + Glu

Cell Viability (%)

Concentration (µM)
PANC-1 in NDM + Glu +AA

Cell Viability (%) vs. Concentration (µM)

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**BxPC-3 in NRM**

**BxPC-3 in NDM**
Cyto-ID Autophagy Detection Kit with Hoechst Nuclear Stain

<table>
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<th>NRM</th>
<th>NRM + 10µM 21</th>
<th>NRM + 50µM CQ + 1µM Rapa</th>
<th>NRM + 10µM 21 + 50µM CQ + 1µM Rapa</th>
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<tr>
<td>NDM</td>
<td>NDM + 10µM 21</td>
<td>NDM + 50µM CQ + 1µM Rapa</td>
<td>NDM + 10µM 21 + 50µM CQ + 1µM Rapa</td>
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*Green Fluorescence* for Autophagosome; *Blue Fluorescence* for Nucleus
Replicate Western blots for effect of 21 on LC3-I & II

RZ 11.7.16

<table>
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<th>Condition</th>
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<th>NRM + CQ</th>
<th>NRM + WM</th>
<th>NDM</th>
<th>NDM + 21</th>
<th>NDM + CQ</th>
<th>NDM + WM</th>
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<tr>
<td>GAPDH</td>
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<td>LC3-I</td>
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<td>17370.6</td>
<td>17396.0</td>
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<td>LC3-I ratio to GAPDH</td>
<td><strong>1.22</strong></td>
<td><strong>1.17</strong></td>
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<td>LC3-II</td>
<td>13895.3</td>
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<td>LC3-II ratio to GAPDH</td>
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<td>LC3-II/LC3-I</td>
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<td><strong>1.50</strong></td>
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LC3-I & II Western Blots
Replicate cytotoxicity assays for 21 and chloroquine