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SYSTEMATIC ANALYSIS OF ANTI-CANCER COMPOUND 6E

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ABSTRACT

The need for novel anti-cancer drugs is constantly under high demand due to the emergence of drug resistance. In order to increase the efficiency of understanding the working mechanism and genetic perturbation of new compounds, network maps are developed to simplify the identification process. These networks correlate the gene expression and biological function of new compounds to those of known drugs based on mRNA expression assayed on DNA microarrays. Based on these maps, novel anticancer compound, 6e, is shown to have similar gene expression with drugs such as thapsigargin tunicamycin, and tosedostat. The preliminary predictions of 6e mechanism are validated using biochemical analysis and imaging. Results showed the induction of cellular stress and the disruption of autophagy leading to cancer cell death by 6e, which corresponds with the working mechanism of thapsigargin and tosedostat predicted by the network maps. 6e can disrupt the progression of autophagy as a prosurvival mechanism, thus preventing cancer cells from escaping from drug induced stress and cell death. 6e is also tested in combination treatments with clinically used drugs. The potential synergistic effects of the drug combinations could lead to the discovery of better treatments for cancer. These results show the possibility of introducing 6e as a novel chemotherapy compound in cancer treatment as well as the prospect of generating networks on a larger scale to simplify the identification of novel drug mechanism.
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Chapter 1

Introduction

1.1 Endoplasmic reticulum stress induction and signaling in cancer

The endoplasmic reticulum (ER) is responsible for many crucial regulations of homeostasis and cellular functions. Disrupted ER functions could be an important factor contributing to the elimination of diseased cells including cancer cells. The ER is the site for many processes including, but not limited to, protein synthesis, protein folding, lipid synthesis, post-translational modifications, gene expression, cellular metabolism, and calcium signaling.\(^1,2\) Under normal conditions, newly synthesized proteins are folded and transported to targeted cellular locations. When pathways within the ER are disrupted due to external or internal events, proteins begin to misfold and accumulate in the ER lumen, inducing ER stress.\(^2,3\) The unfolded protein response (UPR) is an ER stress coping mechanism that can help the ER to reduce the number of misfolded or unfolded proteins.\(^1\) The UPR can either help the misfolded proteins to be refolded into the correct structure or degraded. During ER stress, more misfolded proteins are retained in the ER for further processing by ER protein chaperones until their structures are corrected. Unfolded proteins that cannot be fixed are disposed via ER-associated degradation and eventually elimination of the entire cell.\(^2\) On the molecular signaling level, the UPR consists of three pathways including protein kinase RNA-like endoplasmic reticulum kinase (PERK).\(^3\) PERK is a type I transmembrane ER-resident protein. It has a luminal domain that is
responsible for sensing ER stress. The cytoplasmic portion of PERK contains a protein-kinase domain that can phosphorylate recruited cytoplasmic proteins.\textsuperscript{14} Under normal conditions PERK is in its inactive state by binding with the ER stress sensor binding immunoglobulin protein (BiP). Upon cellular or ER stress, BiP is released from PERK causing the activation of PERK. PERK then autophosphorylates itself in order to phosphorylate and inhibit eukaryotic initiation factor 2α (eIF2α) to shut down protein synthesis, but selectively increasing the expression of activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP).\textsuperscript{1} ATF4 regulates pro-survival genes that are involved in protein folding and cellular stress. The activity of ATF4 can help the ER to reduce the unfolded protein load. In addition, CHOP activation could lead to apoptosis and cell death.\textsuperscript{3} Therefore, ER stress induced signals initiated by PERK eventually lead to cell death via apoptosis and/or autophagy.

Cancer cells also rely heavily on the ER to produce proteins for key signaling pathways. The ER of rapidly proliferating cancer cells is constantly under a heavy workload due to the increased metabolic activities of cancer cells. However, the capacity of the ER to process proteins is limited. As a result, the accumulation of unfolded and misfolded proteins can easily lead to ER stress in cancer cells. Upon ER stress, cancer cells can also employ pathways such as the unfolded protein response and autophagy to maintain ER function similar to the coping mechanism of normal cells. However, due to the excessive production of misfolded or unfolded proteins, ER stress in cancers cells cannot be properly fixed. Consequently, the over-induction of ER stress in cancer cells can cause cell death and elimination. By employing this theory that over stimulation of ER stress will eventually cause cell death despite the coping mechanisms, many
therapeutic drugs for cancer treatment are designed to increase the ER stress level to eliminate cancer cells.\textsuperscript{2,3}
1.2 From cellular stress to autophagy

ER stress is linked to the induction of apoptosis and autophagy. Both apoptotic and autophagic pathways under proper regulation have been shown to be involved in cancer cell elimination and survival. Compare to apoptosis, autophagy is a relatively new cellular process. Autophagy is an intracellular degradation process, which is essential for eukaryotic cell survival. When cells encounter survival stress or nutrient deprivation, they can activate autophagy to degrade and recycle damaged cell organelles to cope with the stress injuries. For example, recycled amino acids from degraded proteins can be used for the synthesis of new peptides in nutrient deprived environments. During autophagy, unique double-membraned autophagosomes are formed to engulf intracellular components. Autophagosomes then fuse with lysosomes to form autophagolysosomes to degrade engulfed components with lysosomal hydrolases.

The specific activation signaling of autophagy has also been extensively studied. Many stress induction pathways such as the PERK- eIF2α-ATF4 system and inhibition of mTOR can lead to the activation of autophagy. In general, proteins involved in autophagy are generally encoded by autophagy related genes, and the identification of autophagy specific proteins such as LC3 is crucial for monitoring autophagy. The activation of the PERK- eIF2α-ATF4 pathway in the unfolded protein response has been shown to upregulate autophagy related gene 12 (Atg12) and converts microtubule-associated protein 1 light chain 3-I (LC3-I) into cleaved form of LC3. LC3 was known to be involved in the regulation of assembly and disassembly of microtubules before the discovery of autophagy. During the formation of autophagosomal membranes, cytosolic LC3 (LC3-I) is converted into intra-autophagosomal LC3-II, and LC3-II is then recruited
to the autophagosomal membrane. Thus, the presence of LC3-II indicates the formation of autophagosomes and the start of autophagy. Through the fusion of autophagosomes with lysosomes, LC3-II is also degraded by lysosomal proteases along with other engulfed components.\textsuperscript{6}

In addition to ER stress induced-autophagy, other cellular stress pathways can also lead to the activation of autophagy. Specifically, nutrient depletion-induced autophagy can activate energy sensor AMP-responsive protein kinase (AMPK) that responds to low levels of ATP. The activation of AMPK then could inhibit autophagy-repressive kinase, and mammalian target of rapamycin (mTOR) to activate of the autophagy-essential Atg1/ULK kinase complex. Hence, the inhibition of mTOR is another major activation route for autophagy in addition to ER stress PERK-eIF2\textalpha mediated autophagy. Target genes for p53 such as Sestrin 1 and Sestrin 2 have also been reported to induce autophagy.\textsuperscript{7} They inhibit mTOR through an indirect mechanism that involves the stimulation of AMPK. On the other hand, autophagy has also been show to be negatively regulated by p53-related pathways. p53 itself can potently inhibit autophagy when it is present in the cytoplasm where the inhibition of AMPK and activation mTOR occurs.\textsuperscript{7}

Autophagy regulation is crucial in cancer cell survival and development. Due to the abnormal cellular activities of cancer cells, the cells are likely to be immersed in stressful environments. In order to cope with the stress, cancer cells can employ autophagy to confer stress tolerance and maintain cancer cell survival. Many drug resistant or recurrent cancers have been reported to show autophagy activation as a method to tolerate drug induced stress. Therefore, blocking or disrupting autophagy in
tumor cells has been suggested in many studies to be an effective treatment for drug resistant cancers. Targeting multiple pre-existing anti-cancer pathways combined with the inhibition of autophagy could potentially be a more effective treatment than targeting a single pathway. The cancer cells are not only killed by the drug induced stress, but also are unable to escape from stress. As a result, more cancer cells can be eliminated from an organism. Combination of autophagy inhibitors with clinically used chemotherapies such as imatinib, a Bcl/Abl inhibitor, showed promising outcomes when applied to patients with relapse leukemia.⁸
1.3 6e as a novel anti-cancer compound

Resistance to chemotherapies is a major obstacle in cancer treatment. Therefore, the discovery of effective and harmless anticancer agents would be beneficial in the battle against drug resistant cancers. Studies have shown many naturally occurring chemicals, such as indole alkaloids and imidazoline, exhibit anticancer activities. Toxicity or side effect observed in indole alkaloid and imidazoline compounds is mainly due to the variation in side groups or conjugates attached to the main structure. Fumitremorgin C, a natural fungal product, belongs in the indole alkaloids family that presents selective inhibition of breast cancer resistance proteins. However, this compound also induces tremors and cell cycle arrest when used as a chemotherapy agent. Therefore, the search for modified compounds similar to fumitremorgin C but without the harmful side effects allowed the discovery of many new anticancer agents. These compounds are synthesized to have different conjugates or side groups attached to the structure backbone so that the new anticancer agents are safer for clinical usage than the parent compounds. 6e is one of those compounds that exhibits high anticancer activities in many cancer cell lines and low toxicity. The structure of 6e contains a valine-O-benzylester and a 4-H-b-carboline group (Figure 1). The valine side chain of 6e is important for its anticancer activity while the carboline group provides the compound with a fluorescent tag that can be used for visualization (Figure 2). Based on the preliminary analysis of 6e’s physical properties and morphology, 6e was observed assemble into small particles when dissolved in water. When 6e was added to cell culture, the small particles of 6e can then adhere to the cell membrane and enter the cell via endocytosis (Figure 2). Specific side groups on the 6e molecules are crucial for its anti-cancer activity. Altering the valine side group prohibited
the anticancer activity as well as the specific morphology properties of 6e. The self-fluorescent property of 6e provides a useful characteristic for visualizing the compound in experiments using the compound.
1.4 Chemical inducers of the unfolded protein response, ER stress, and autophagy

Many chemicals and drugs have been developed to regulate specific pathways such as ER stress or autophagy in cells when normal cellular functions are compromised. Thapsigargin and tunicamycin are two conventional ER stress inducers that are commonly used in research and clinical settings.\textsuperscript{9,10} Thapsigargin is an inhibitor of the ubiquitous sarco-endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) in eukaryotic cells. Thapsigargin can regulate Ca\textsuperscript{2+} binding, signaling and storage in the ER.\textsuperscript{9} The release of stored Ca\textsuperscript{2+} from intracellular compartment by thapsigargin has been shown in many studies.\textsuperscript{9,10} The inhibition of SERCA by thapsigargin prevents the homeostasis of Ca\textsuperscript{2+} between active pumps and passive leaks of Ca\textsuperscript{2+}. This disruption of calcium signaling is a major factor that could contribute to ER stress. Therefore, thapsigargin induces ER stress via SERCA inhibition and calcium signaling disruption. Recent studies have also shown thapsigargin can be used as an autophagy inhibitor.\textsuperscript{9} Specifically, thapsigargin blocks the fusion of autophagosomes with lysosomes during autophagy while leaving the initiation of autophagy and endocytic system functional. Thapsigargin treatment does not inhibit the induction of autophagy or autophagosomes formation. As a result, autophagosomes are still observed in thapsigargin treated cells with autophagy marker LC3-II present on autophagosomes. Following the initiation of autophagy, thapsigargin then causes clustering of autophagosomes and disrupts the recruitment of the fusion machinery with lysosomes.\textsuperscript{9} The inhibition of autophagolysosome formation prevents the continuation of autophagy. Cells can die faster since the pro-survival process of autophagy is inhibited.

Tunicamycin, another widely used ER stress inducer, is a bacterial toxin that inhibits N-linked glycosylation of proteins resulting in the activation of UPR in
eukaryotic cells. Specifically, the inhibition of N-acetylglucosamine transferase and the prevention of the formation of N-acetylglucosamine lipid intermediates by tunicamycin initiate the signaling of ER stress. As a result tunicamycin exhibits effective antibacterial and antifungal activities.\textsuperscript{11}

Although specific autophagy inhibitors and inducers have been developed over the years, some commonly used drugs for other diseases have also been shown to affect autophagy regulation. Chloroquine is commonly used as an antimalarial drug. Despite its involvement in eliminating malaria-causing parasites, it can also block lysosomal acidification and degradation of autophagosomes.\textsuperscript{12} As a result, chloroquine is an autophagy inhibitor. This property of chloroquine has been shown to exhibit anti-cancer effects in several cancer cell lines including breast and colon cancers.\textsuperscript{8} Current chemotherapy treatments frequently induce prosurvival autophagic pathways as the drugs themselves present cellular stress to the targeted cancer cells. As a result, many chemotherapeutic agents have been shown to be less effective due to the stress coping mechanism used by cancer cells. In order to create better treatment, the combination of autophagy inhibitor, such as chloroquine, with currently used chemotherapeutic agents shows promising results in treating cancer.\textsuperscript{8,12} Many clinical trails have been established to examine the effects of chloroquine combine with other drugs. Although drug resistance is becoming a major concern for many chemotherapy plans, the emergency of synergist effects of drug combinations presents promising solutions.

The purpose of this study is to perform a systemic analysis of 6e in an attempt to understand the working mechanism of this novel anti-cancer compound. Based on gene expression perturbations and biochemical analysis, the general drug mechanism of 6e
could be predicted. The understanding of 6e mechanism can potentially help the development of more effective treatments to overcome drug resistant cancers. Either by using 6e alone or combining 6e with other clinically used chemotherapy drugs, the possibility of introducing another anti-cancer therapy presents many beneficial influences to medical research.
Chapter 2

Material and Methods

2.1 Cell culture and drug treatment

Human osteosarcoma U2OS and multiple drug resistant human uterine sarcoma MES-SA/Dx5 cells were cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin in a 5% CO₂ 37°C incubator. Once cells have reached 60%-80% confluency, drug treatments were applied. Concentrations and duration of 6e treatment or combination treatments were performed as specified in the figure legends and text of the current study.

2.2 ATP Luminescence Assay

U2OS cells were plated in 96-well plates with a cell count of approximately 2,000-3,000 cells per well. 100µL fresh medium was added to each well prior to drug treatment. Various concentrations of 6e or combined drug treatments were added to each well as specified in the figure legends and text. The cells were then placed in a 37°C incubator with 5% CO₂ for 48 hours. The ATP luminescence assay was performed after 48 hours of incubation following the manufacturer’s instructions for CellTiter-Glo® Luminescent Cell Viability Assay.
2.3 mRNA extraction and qPCR

RNA was extracted using the RNeasy Mini Kit per manufacturer’s instruction. 1 µg RNA was reverse transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences) per manufacturer’s instructions. Quantitative PCR was performed with various primers using SYBR Green SuperMix (Quanta Biosciences) in the StepOnePlus Real-Time PCR System (Applied Biosystems).

2.4 Microarray

U2OS cells were treated with 30 µM 6e dissolved in DMSO for 8 hours before harvesting. Control cells were mock treated with DMSO. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) per manufacturer’s instructions. The Affymetrix GeneChip Human Gene 1.0 ST array was used for expression microarray. RNA labeling, hybridization, scanning, and data acquisition were done at the Genomics Core Facility at the Pennsylvania State University. Data gathered were then analyzed using Ingenuity® pathway analysis (IPA) program. 6e microarray results were cross-referenced with the IPA database to generate a list of all chemicals, drugs, and compounds that had common genes compared with 6e microarray results.

2.5 Construction of network maps

The microarray data generated by IPA was compiled as a list in a Microsoft Excel file. The 15 chemicals and drugs were manually selected based on p-value correlation to novel compound 6e. Information gathered from the microarray analysis including p-value, z-score, and specific gene targets were depicted in a network map using Adobe Illustrator.
p-value was transformed into line density that connected the ovals in the maps. Z-score was reflected as the coloration of the ovals. The biological function of each of the 15 drugs was identified using publications and Internet resources. The common gene targets identified by IPA were used to create Venn diagrams in Adobe Illustrator. The top three compounds with the highest correlation were used to generate the diagram.

2.6 Protein extraction and Western Blot

Western blotting was performed following protein extraction from target cells. Cells were harvested by re-suspension in an appropriate volume of cold IP buffer (10mM EDTA, 2mM Tris-HCl pH 8.0, 150mM NaCl, 0.2% Triton X-100, 0.2% NP-40) supplemented with protease inhibitors (10µg/µL PMSF, 1µg/µL Leupeptin, 1µg/µL Aprotinin, 1µg/µL PepstatinA). Mixture was then sonicated for ~5 min at 4 °C followed by SDS denaturation. The appropriate volume of denatured protein was loaded into a 12% SDS-Acrylamide Gel (2.15mL ddH₂O, 4.6mL 30% Acrylamide, 4.1mL 1M Tris-HCl pH 8.8, 100µL 10% SDS, 100µL 10% APS, 3.8µL TEMED) and run at 220V for 45 minutes. Protein was then transferred to Nitrocellulose membrane, using a Semi-Dry Transferring system for 1 hr. Following Ponceau S staining, the membrane was blocked in 5% Milk Tris-Buffered Saline and Tween 20 (TBST) for approximately 30 minutes at room temperature to which the following primary antibodies were added: PERK, eIF2α, eIF2α-pS51, ATF4, DDIT3, DDIT4, HO1, Hsp70, SESN2, and LC3 at suitable dilutions. Actin was used as a control. Following overnight incubation at 4°C, membranes were washed three times in TBST for 10 min. each and were then incubated for 2 hrs. at 4°C with the
proper secondary antibody. Signals were detected using the Lumi- Light PLUS Western blotting substrate (Roche).

2.7 Immunostaining and Fluorescent Imaging

Immunostaining was performed using a previously established protocol. After fixation of samples with 3.7% paraformaldehyde in phosphate buffered saline (PBS) supplemented with 1% Triton X-100 and 2% NP-40, cells were washed with PBS three times for 10 min each. Following the third wash, cells were blocked in 2% BSA in PBS for at least 2 hours at room temperature. Primary antibodies were diluted in PBS supplemented with 2% BSA and 5% normal goat serum as follows: 1:50 of LC3 and 1:400 for each of α and β microtubule. Cellular staining was performed in a humid chamber overnight at 4°C. After application of the primary antibodies and overnight staining, the cells were washed with PBS three times for 10 min each. Cells were then stained with the appropriate secondary antibodies conjugated with Alexa488 at a 1:500 dilution for 2 hours at room temperature. After washing three times for 10 min each with PBS, cells were stained with 1µg/µL Hoeschst for 15 seconds followed by a final wash with dH2O. Slides were then mounted and imaged with a confocal microscope. Images were later processed and adjusted using Adobe Photoshop as appropriate.
Chapter 3

Results

3.1 Network map prediction of 6e mechanism

Novel compound 6e was shown to exhibit anticancer activity in various cancer cell lines such as osteosarcoma U2OS cells (Figure 3). 6e presented high killing efficacy at relatively low concentrations. In order to further understand the working mechanism and the potential clinical usage of 6e, microarray analysis of 6e was cross-referenced with IPA database to compare with known drugs and chemicals. Based on IPA analysis, a network map was developed for 6e to predict a preliminary working mechanism. IPA generated a list with all the chemicals and drugs that shared common upstream gene perturbations as 6e. The top 15 compounds with the highest correlation were used to generate the network map (Figure 4A). The correlation was suggested by p-value. A small p-value showed 6e and the known chemical would cause similar gene expressions changes in cells. Tosedostat had the highest correlation with 6e with a p-value of $4.49 \times 10^{-27}$, which was reflected via the weight of the line connecting to 6e. Palmitic acid had the lowest p-value in the list of top 15 compounds. Z-score is another value used to reflect relationship of 6e with known compounds. The z-score value indicated the regulation direction when compared with 6e. Positive values indicated the same regulation scheme whereas a negative value indicated the opposite. Many of the 15 compounds used had positive z-scores, which were shown with the red oval. Compounds such as PD9805 and SP600125 were the only two in the list that had negative z-scores,
which meant if 6e were upregulation a set of genes, these two compounds would
downregulate the same set of genes. In addition, the main biological function of each
compound was also identified and illustrated in the gray ovals. Based on the IPA list
generated, tosedostat, tunicamycin and thapsigargin were the top three drugs with the
highest correlation. The specific genes targets that overlapped with 6e for these 3
compounds were also identified by IPA, and these identified genes were used to generate
a Venn diagram to illustrate the overlapping genes (Figure 4B). Expression of ASNS,
ATF3, CEBPB, DDIT3, and DDIT4, were significantly altered for all three drugs.
3.2 6e treatment induced cellular stress

Due to the high correlation of 6e with conventional ER stress inducers, such as thapsigargin and tunicamycin, further biochemical analyses were performed to determine the involvement of 6e in ER stress. By using ER stress pathway markers such as PERK, eIF2α, eIF2α –phosphorylated, ATF4, and CHOP, the involvement of 6e in these pathways was determined. Upon the stimulation of stress signals, unfolded proteins would accumulate in the ER to cause ER stress. ER stress triggers the activation of PERK that phosphorylates eIF2α. The phosphorylation of eIF2α could then lead to the activation of other downstream ER stress-related genes directly involved in the initiation of cell death. Results showed elevated expressions of the unfolded protein response sensors and ER stress related genes at both transcription and protein levels (Figure 5A and 5B). As the concentration of 6e increased from 0µM to 15µM to 30µM, the level of UPR sensor and ER stress markers also significantly increased. In addition, decreased cell growth and increased number in dead cells was observed in cell culture plates with increasing 6e concentrations. These findings suggest that ER stress could be an important factor contributing to 6e mediated cancer cell death.

In addition to the PERK- eIF2α-ATF4 signaling pathway, the inhibition of mTOR pathway was also likely to be involved in 6e-induced stress. Increased levels of Sestrin2 (SESN2) was observed (Figure 6A and 5B). SESN2 is a p53 target gene that has been reported to induce autophagy. Both mRNA and protein levels of SESN2 were notably greater in cells treated with 6e than those of control cells. Increasing the concentration of 6e from 15 µM to 30 µM significantly changed the expression level of SESN2 at both mRNA and protein expression levels.
3.3 6e induced cellular stress-mediated macroautophagy disruption

In order to better understand the downstream killing mechanism of 6e, further testing was performed. Based on findings in the literature and network map predictions, it was thought that 6e-induced cell death could involve autophagy. In addition, evidence has shown to support the idea that various cellular stresses can lead to the induction of autophagy. Specifically, ER stress and nutrient deprivation can all trigger to the initiation of autophagy as cells cope with the induced stress. The increased levels of ER stress marked by the PERK- eIF2α-ATF4 signaling pathway and mTOR inhibition marked by increased levels of SESN2 can all cause autophagy.

Additionally, results assayed on mRNA and protein expression levels showed an increase in autophagy marker, LC3. LC3 is an important biomarker for the progression of autophagic stages. Gene expression of LC3B was significant greater in 6e treated U2OS cells than in control cells (Figure 6A). After transcription and translation, LC3-II is a protein that locates on the membrane of autophagosomes following the modification of cytosolic LC3-I. Western blot results showed a significant increased level of LC3-II in 6e treated cells. In control cells, there was no LC3-II expression (Figure 6B). Immunostaining results also validated the accumulation of LC3-II on a cellular level (Figure 6C). Clusters of LC3-II were observed in significantly greater amounts in 6e treated U2OS cells. Results from gene expression, protein expression, and cell imaging all support the idea that 6e induced accumulation of LC3II and the initiation of autophagy.
3.4 Potential synergistic killing effects of 6e with chloroquine

In order to find a more effective use of 6e, the synergist killing effects of 6e was tested. Chloroquine was chosen for the combination treatment since chloroquine has been identified to disrupt autophagy.\textsuperscript{12} Results showed the combination treatment of 6e with chloroquine significantly increased killing efficacy in both U20S and MES-SA/Dx5 cancer cell lines. In U2OS cells, approximately 70% cells were still viable after 16\textmu M 6e only treatment. Approximately 50% cells were alive after 16\textmu M chloroquine only treatment in U2OS cells. The combination of 6e and chloroquine treatment with a 1:1 ratio at 16\textmu M each killed approximately 80% of the initial population (Figure 7A). Similar pattern of cell killing was also observed in MES-SA/Dx5 cell line (Figure 7B). The combination treatment of 6e and chloroquine was significantly more effective at eliminating cancer cells than individual treatments. In both cell lines, 16\textmu M of each drug in the combination treatment was able to eliminate more than 80% of the cells.
Chapter 4

Discussion

Due to the emergence of drug resistant cancers, the need for novel anti-cancer drugs is constantly under high demand. Novel compound 6e has shown promising killing effects against many cancer cell lines and not normal cells, which presents the potential for clinical usage. 6e was tested on multiple normal cell line including epithelia cells, and no significant killing was observed (data not shown). In order to understand the drug working mechanism of 6e, a systematic analysis was employed. Microarray results and biochemical analyses provided insight into the mechanism of 6e. Drug treatment of 6e could first induce cellular stress via many systems such as the ER stress induced unfolded protein response. As a coping mechanism to 6e-induced stress, the cells initiate autophagy in an attempt to recover from the stress. 6e then could inhibit and disrupt the progression of autophagy to prevent cancer cells from coping with the drug-induced stress. Based on current understanding of the compounds, 6e could potentially lead to the disruption of autophagy and induction of cellular stress via two methods. The first is that 6e could enter lysosome via endocytosis to disrupt the degradation of unwanted cellular components causing ER stress and fusion with autophagosome during autophagy causing autophagy inhibition. The second possible binding system of 6e after entering the cells is via mitochondria disruption, which could lead to apoptosis and the disturbance of cellular functions such as energy production. Despite the effects of 6e treatment alone, results also showed potential synergistic killing effects when 6e was combined with chloroquine.
Overall, 6e-induced stress and the inhibition of autophagy caused significant decrease in cancer cell population. The combination treatment proposed another possible applicable use of 6e in clinical settings.

After 6e treatment showed effective killing rates on various cancer cell lines, the identification of specific pathways that 6e could be involved in was investigated. Microarray analysis of 6e treated U2OS cells was performed in order to obtain a preliminary identification of genes regulated by 6e. Results were specifically analyzed using Ingenuity® pathway analysis to cross-reference 6e treated cell gene perturbation with those of known drugs or chemicals in the IPA database. This IPA analysis provided a list of hundreds of chemicals and drugs that were correlated with 6e. It was thought that based on the drug mechanism of highly correlated chemicals, the identification of 6e mechanism would be simplified and better targeted. By using the top 15 chemicals or drugs given by the IPA list, network maps were manually generated to visualize the results. Gene perturbations caused by tosedostat, tunicamycin, and thapsigargin overlapped best with 6e. The correlation value, directionality of regulation, function, and the specific common genes shared among these drugs are illustrated in the network maps (Figure 4A and 4B). Based on previously published literature, tosedostat, tunicamycin, and thapsigargin all induce cellular stress mediated cell death. Tunicamycin and thapsigargin specifically are typically used ER stress inducers that cause apoptotic or autophagic cell death.9,11 Additionally, thapsigargin has been shown to disrupt autophagy after the induction of autophagy activation.10 Tosedostat is an anti-cancer compound that is currently used in many clinical trials against acute myeloid leukemia.13 Based on the information gathered on these drugs with the highest correlation to 6e, it was predicted
that 6e was very likely to be involved in ER stress pathways and the following
downstream cell death pathways. The use of these network maps significantly narrowed
down the search for possible mechanism involved in 6e induced cell death, which
allowed a more targeted biochemical analysis.

Biochemical analysis of mRNA and protein expression level on ER stress related
genes were extensively examined. Results showed 6e treated osteosarcoma cells U2OS
were overexpressing ER stress and unfolded protein response marker genes such as
PERK, eIF2α, and ATF4 (Figure 5A and 5B). Based on these results, the involvement of
6e in ER stress and unfolded protein response was confirmed. After 6e enters a cell, the
drug is likely to bind with cellular components to induce ER stress and the accumulation
of unfolded or misfolded proteins in ER lumen. This abnormal condition in the ER would
lead to a series of signaling that eventually activates apoptosis or autophagy as the cells
try to manage the stress.

In order to determine the specific downstream mechanism that can directly cause
cell death, additional experimentation was performed targeting the process of autophagy.
Due to the correlation of 6e with thapsigargin, the induction of autophagy was tested on
6e treated U2OS cells. Results revealed the accumulation of autophagosome marker
LC3II, indicating the activation of autophagy due to 6e. The increased expression level of
LC3II was observed on mRNA, protein, and cellular level, which further confirmed the
induction of autophagy by 6e. In addition, both ER stress and nutrient deprivation
mediated mTOR pathway marked by SESN2 can induce the initiation of autophagy.
However, since autophagy has been known as a prosurvival pathway for cells to cope
with cellular stress, 6e was thought to disrupt autophagy after activation, similar to
thapsigargin. As stated before, thapsigargin, a highly correlated drug with 6e, has been shown to initiate autophagy first, and then inhibit the completion of autophagy by disrupting the fusion of autophagosome with lysosome. Increased levels of LC3II can also be observed in thapsigargin treated cells similar to the results gathered for 6e. Therefore, it is probable that 6e initiate autophagy due to cellular stress, but then disrupts the progress by inhibiting the formation of autophagolysosomes. The high levels of LC3II could also be the result of disrupted autophagy since fusion with lysosomes is inhibited. If fusion of autophagosomes and lysosomes can occur, proteasomes in lysosomes will degrade the LC3II on the autophagosomes leading to a decreased level of LC3II. Without degradation, LC3II increase should be more prominent, which was observed in the results. Further experimental results on this inhibition of autophagosome fusion with lysosome were obtained by another lab member, which confirmed the prediction that 6e disrupts autophagy based on the correlation of 6e with thapsigargin. The experiments performed were based on the pH difference of autophagosome and lysosome. Due to the acidic environment of lysosomes and neutral pH of autophagosome, the fusion progress can be tracked during autophagy. Results from these experiments showed the disruption of autophagy by 6e as the drug inhibits the fusion of autophagosome and lysosome.

Overall, the general working scheme of 6e starts with the initiation of multiple cellular stresses including ER stress, and unfolded protein response. The stress signal can cause cell death such as apoptosis as well as induce cellular coping mechanism such as autophagy. The progression of autophagy can then be inhibited by 6e so that the cancer cells cannot escape from the induced stress (Figure 8). In cancer cells, the activation of
autophagy is a used as a pro-survival pathway to avoid cell death. By inhibiting autophagy, cells are more likely to be destructed. Therefore, the induction of cellular stress together with inhibition of autophagy makes 6e an effective compound at killing cancer cells.

In order to further increase the killing effects of 6e, combination treatment of 6e with chloroquine was performed on two different cancer cell lines. Results showed potential synergistic killing effects. Since both chloroquine and 6e inhibit autophagy, the combination of the two would promote more potent effects on the cells than any single drug treatment. Despite chloroquine’s anti-malaria effects, chloroquine has been used in many clinical trails in combination with other drugs to treat relapse cancers. The synergistic killing effects exhibited by combining 6e with chloroquine provides another aspect that showed 6e could have clinical potential.

In conclusion, novel compound 6e showed significant anti-cancer effects similar to many currently used drugs. Although the exact mechanism of 6e is still under investigation, the compound exhibits promising features that could be employed in the clinical market one day. Furthermore, the use network maps generated from microarray results analyzed by IPA can potentially simplify the mechanism identification process of novel compounds. The network maps can provide a preliminary prediction of the novel drug mechanism by comparing gene perturbation with stored data for thousands of drugs and chemicals. The comparison can narrow down the search to a few highly correlated pathways.
**Figures**

**Figure 1. 6e structure**

The chemical structure of 6e is depicted in Figure 1. The highlighted group in red retains the anti-cancer property of 6e. The fluorescent moiety allows the compound to be visualized by fluorescent microscopy.

**Figure 2. 6e morphology in cells**

The left panel shows 6e morphology in a U2OS cell observed under phase contrast microscopy. The red arrows point to the 6e particle formation on the surface of the cell and inside the cell. The right panel shows the same cell observed under fluorescent microscopy. The green fluorescent particles are 6e on cell membrane and various cell organelles. The self-fluorescent property of 6e allows the compound to be visualized without the need for additional dyes.
**Figure 3. 6e killing curve**

U2OS and MES-SA/Dx5 cells were treated with different concentrations of 6e for 24 hours. The killing efficacy of 6e was determined by using ATP luminescence assay. The IC_{50} for 6e was determined to be ~7\mu M for U2OS cells and ~15 \mu M for MES-SA/Dx5 cells. Three repeats for each experiment was performed. The error bars reflect the positive and negative standard deviation values of each data point.
Figure 4. Network maps of 6e

Figure 4A shows the correlation of 6e with 15 known compounds based on \( p \)-value gathered from microarray data. Weights of the lines connecting 6e with known chemicals were determined by using \( -\log(p\text{-value})/5 \). Thicker lines showed stronger correlation of 6e with known chemicals based on overlapping genes. Oval colors reflected the Z-score of each known compound. A negative Z score (green) value indicates the opposite regulation scheme of 6e and the known chemical. A positive Z score (red) value indicates a similar regulation scheme of 6e and the known chemical. Gray ovals show the main function of the 15 chemical compounds. Figure 4B shows the specific genes targets for the top three chemicals with the highest correlation to 6e and the overlap of the genes.
Network Maps systematically predict novel drug mechanisms. These maps correlate the gene expression and biological function of a new compound to those of 15 known drugs with the smallest molecular perturbation of new compounds with potential for clinical usage, gene expression network maps were developed to match the top three chemicals with the highest correlation based on the network maps. eIF2a showed decreased globular translation level. The significant increase of DDIT3 indicated the cells have a significant increase in UPR genes was detected in both mRNA and protein level. Increasing phosphorylation of ATF4 was detected in both mRNA and protein level. (A) mRNA and protein level changes of UPR genes with treatment of 6e. (B) Western blot of PERK, eIF2α, eIF2α-pS51, ATF4, DDIT3/CHOP, DDIT4, HO1, Hsp70, SESN2, and Actin with treatment of 6e.
Figure 5. 6e treatment induced cellular stress

In U2OS cells treated with 6e, significant increases in unfolded protein response, ER stress, oxidative stress, and nutrient deprivation markers were detected in both mRNA and protein expression. The A) real-time PCR results and B) Western results reflected such change.
Network Maps

smallest systematically predict novel drug mechanisms. These maps correlate the gene expression and biological function of a new compound to those of 15 known drugs with the smallest

The discovery of novel drugs for cancer treatment is under high demand due to the emergence of drug resistance. In order to accelerate drug discovery, a new technique was developed to screen for molecules with potential utility in cancer. Gene expression network maps were constructed to compare the molecular perturbation of new compounds with potential for clinical usage to those of known chemicals. The significant increase of DDIT3 indicated the cells have

Fatty Acid

Tunicamycin

Expression levels relative to actin

synthesis

Peptidase

Tosedostat

SP600125

Z-Score:

Tosedostat

MEK1

SEC61A1

SDF2L1

PTGS2

PP1R15

NDRG1

ERO1LB

EDEM1

compound compare to the gene regulation of known chemicals. Oval colors of the known chemicals reflected the Z score of gene regulation of each chemical. A negative Z score value (red) indicated similar gene regulation of the novel chemical to the novel chemical.
Figure 6. 6e treatment and autophagy

U20S cells were treated with various concentrations of 6e. Figures 6A and 6B showed altered mRNA and protein levels indicating the initiation of autophagy induced by cellular stress signal SESN2. Figure 6C showed immunostaining microscopy images that indicated the presence of autophagy marker LC3 in 6e treated cells. Arrows indicate specific cluster of LC3 proteins in 6e treated cells that are not observed in control cells. Microtubule staining was also performed to visualize cell shape and autophagosome structure. Microtubule arrangement also seems to be disturbed in 6e treated cells.
**Figure 7. Synergist killing effects of 6e with chloroquine**

6e and chloroquine was combined with a 1:1 ratio at various concentrations. U2OS (Figure 7A) and MES-SA/Dx5 (Figure 7B) cell lines were treated with the combination of 6e and chloroquine and individual drugs. The combination treatment showed synergistic killing effects as more cells were killed at a lower concentration of combined treatment than that of the individual drug treatments. Three repeats were performed for each experiment. Error bars reflect the standard deviation calculated for each data point.
Cellular Stress

- Oxidative Stress
- ER Stress
- Nutrient Deprivation

Unfolded Protein Response

- PERK
  - eIF2α
  - eIF2α

- ATF4
- CHOP

Apoptosis/Cell Death

Autophagy

- Autophagosome
- Lysosome
- Autophagolysosome

- HO1
- SESN2
- ER membrane
- p

mTOR

- 6e
- Cell membrane
**Figure 8. 6e Mechanism Overview**

The figure illustrates the overview of the 6e mechanism in inhibiting cancer cell growth. 6e can induce multiple cellular stresses such as oxidative stress, ER stress, unfolded protein response, and nutrient deprivation. The cancers cells can activated various signaling pathways responding to the 6e induced cellular stress signals. As a result, the cells either commit to stress coping mechanism such as autophagy or cell death. Autophagy can then be inhibited by 6e to prevent cancer cells escaping from cell death. 6e can inhibit the fusion of autophagosomes with lysosomes as the drug disrupts the progress of autophagy.
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