CHOLANGIOCARCINOMA COMBINATION THERAPY: TAMOXIFEN AND GEMCITABINE PRODUCE AN ADDITIVE EFFECT IN VITRO DUE TO DIFFERING MECHANISMS AND WILL BE AN EFFECTIVE COMBINATION IN VIVO

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CHOLANGIOCARCINOMA COMBINATION THERAPY: TAMOXIFEN AND GEMCITABINE PRODUCE AN ADDITIVE EFFECT IN VITRO DUE TO DIFFERING MECHANISMS AND WILL BE AN EFFECTIVE COMBINATION IN VIVO

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BASIC MEDICAL SCIENCES

ABSTRACT

Cholangiocarcinoma, a fatal tumor arising from the biliary epithelium, has a very poor 5-year survival rate due to the lack of early diagnosis and effective therapies. Stimulation of the Fas-mediated and mitochondrial apoptosis pathways are promising therapies for this tumor. CaM antagonists such as Tamoxifen (TMX) induce apoptosis of cholangiocarcinoma cells in a Fas-dependent manner. The studies presented in this thesis were designed to analyze the apoptotic effects of TMX and a currently used therapy for cholangiocarcinoma, Gemcitabine (GMC), as a combination therapy for cholangiocarcinoma.

A cholangiocarcinoma cell line, sk-cha-1 was found to have two populations, one that expresses a high level of surface Fas (termed here as Fas High), and another which expresses Fas a lower level of (Fas Low). Fas High and Low cells were treated with 1μM to 10μM Gemcitabine and 5μM to 20μM Tamoxifen. Fas High cells were highly sensitive to both therapies with 32% and 33% of the cells undergoing apoptosis after treatment with 15μM TMX and 5μM GMC respectively (n=3, p≤0.05). In contrast Fas Low cells were unresponsive to TMX treatment but fully responsive to GMC. Combination treatment with GMC + TMX in Fas high cells induced apoptosis in an additive manner with TMX-23.8±0.8%; GMC- 31.5±2.8%; GMC+TMX-47.2±3.9%.
To elucidate the different mechanisms used by each drug, caspase activity assays were employed. TMX activated primarily caspases 8 and 10, whereas GMC activated caspases 8, 9 and 10. GMC but not TMX stimulated cytochrome c release into the cytosol. Cell cycle analyses showed that GMC alone caused cell cycle arrest. Tamoxifen and Gemcitabine are shown here to produce an additive effect on apoptosis in vitro by utilizing two different mechanisms, which is currently being tested in vivo. A positive outcome in the animal experiments is expected to lead to clinical trials.

Keywords: cholangiocarcinoma, apoptosis, tamoxifen, gemcitabine
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INTRODUCTION

Cholangiocarcinoma

Cholangiocarcinoma is an uncommon, highly malignant, fatal tumor which arises from cholangiocytes along the intrahepatic or extrahepatic biliary tree. Most cases of cholangiocarcinoma occur sporadically and the exact etiology is largely unknown. There is evidence that chronic inflammation and biliary duct cell injury induced by the obstruction of bile flow are two of the main conditions associated with the development of cholangiocarcinoma (1). Prognosis of cholangiocarcinoma is generally poor with a 5-year survival of less than 5% without surgery (2). A cure can only be expected from surgical resection of early stage tumors. After surgery, the 5-year recurrence rate is in the range of 60-90%. However, most patients present initially with advanced stage disease which is not amenable to surgical cure, and 75% of patients die within 1 year of diagnosis (3).

Due to the advanced stage that most patients are diagnosed in and the lack of effective therapies, cholangiocarcinoma is a very lethal disease (4). Understanding the pathogenesis and progression of cholangiocarcinoma is necessary to develop new ways for detection at an earlier stage, preventative therapies and treatment for patients who already have cholangiocarcinoma. Cholangiocarcinoma cells possesse a resistance to apoptosis that has been proposed to be one of the important mechanisms in its pathogenesis (5). Apoptotic regulators, such as Fas (6), FasL (6), FLICE like inhibitory protein (FLIP) (7), B-cell lymphoma/leukemia 2 (Bcl-2) (8), cyclooxygenase-2 (COX-2)
(9), protein kinase B/AKT (10) and Ca2+/Calmodulin (11) have been studied as potential preventative and therapeutic targets in cholangiocarcinoma (12). The Fas pathway, one of the well characterized avenues for extrinsic apoptotic death, may provide a novel avenue for early diagnosis and possibly effective therapy for cholangiocarcinoma. There is a reported relationship between the expression of surface Fas receptors and disease progression in cholangiocarcinoma patients such that low Fas expression is correlated with a more advanced disease, suggesting its use as a potential marker of disease progression (13). Targeting the Fas pathway in cholangiocarcinoma cells inhibits their growth, and induces apoptosis indicating its potential as a target for therapy. Calmodulin (CaM)-antagonists, such as Tamoxifen (TMX) and Trifluoperazine (TFP), have been shown to induce apoptosis in cholangiocarcinoma cells in a Fas-dependent manner and also inhibit cholangiocarcinoma tumorigenesis in nude mice xenografts, indicating that they may be agents for chemotherapy (14).

Apoptosis

Apoptosis (programmed cell death) plays an important role in embryogenesis, metamorphosis, tissue atrophy, tumor regression, and immunity (15). A homeostatic balance is maintained between cell proliferation and cell death. Too much or too little apoptosis disturbs this balance, leading to various diseases either characterized by cell loss, such as AIDS and neurodegeneration or diseases characterized by failures in apoptosis or an accumulation of cells such as cancer (16).

Apoptotic cell death is characterized by controlled autodigestion of the cell. Cells initiate their own apoptotic death through the activation of endogenous proteases. This
results in cytoskeletal disruption, cell shrinkage, and membrane blebbing. The nucleus undergoes condensation as endonucleases are activated and begin to degrade the cell’s DNA. The dying cell maintains its plasma membrane integrity, however, alterations in the plasma membrane of apoptotic cells signal neighboring phagocytic cells to engulf them to complete the degradation process (17). Cells not immediately phagocytosed break down into smaller membrane-bound fragments called apoptotic bodies. An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response. In contrast, necrotic cell death is associated with an early loss of cell membrane integrity, resulting in leakage of cytoplasmic contents and the induction of an inflammatory response (18).

Apoptosis can be initiated through a variety of triggers such as surface death receptor ligands, chemotherapeutic agents, infections, and stress. The cause of apoptosis varies but the basic machinery used and the resulting cellular morphology remains the same. There are two main pathways that can lead to the end result of apoptosis; one of the pathways (extrinsic) occurs through death receptors stimulation, and the other (intrinsic) utilizes the mitochondria to carry out apoptosis.

**Extrinsic apoptotic pathway**

The extrinsic pathway is activated by ligand-bound death receptors including TNF-TNFR1, FasL-Fas and TRAIL-DR4 or -DR5. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily and generally can have several functions including initiating apoptosis (19). The TNFR superfamily is characterized by the presence of cysteine rich domains that mediate binding between ligands and their type
I transmembrane domain receptors. Within this superfamily, the death receptors including TNFR1 (TNF receptor-1), CD95 (or Fas) and the TRAIL receptors DR4 and DR5 are best characterized. Ligand-bound-receptor can induce a conformational change and/or allow the recruitment of adapters that form higher-order receptor complexes (20). Death receptors and adaptors become activated in the death-inducing signaling complex (DISC) so that they can bind the effector molecules such as procaspase 8 to induce the activation of the downstream apoptotic signaling pathway (21).

**Fas pathway**

Fas has a central role in the regulation of apoptosis and has been implicated in the pathogenesis of various malignancies (22). Some types of tumors have been reported to express FasL, which may be a mechanism to evade attacking lymphocytes (23). Mutations in FasL and Fas have been found in children with autoimmune lymphoproliferative syndrome (ALPS) or Canale-Smith syndrome (24).

The death-inducing signaling complex (DISC) was first described in the FasL-Fas apoptotic signaling pathway (25). The binding of Fas to the Fas ligand promotes receptor multimerization that results in intracellular clustering of death domains (DD). This allows an adaptor protein called FADD (Fas-associated death domain) to associate with the receptor through an interaction between homologous death domains. FADD also contains a death effector domain (DED), which allows binding of pro-caspase 8 to the Fas-FADD complex (26). Pro-caspase 8 (also known as FLICE) associates with FADD through its own death effector domain. Caspase 8, the main initiator caspase in Fas-mediated signaling, is expressed as two isoforms, caspase 8/a and 8/b, which are both recruited to
the activated CD95 receptor (27). FasL-induced clustering of Fas, FADD, and caspase 8 within the DISC leads to the autoproteolytic processing of caspase 8 by induced proximity and dimerization, this leads to the release of the processed active proteases. Following its autoproteolytic cleavage, caspase 8 is released from the DISC as an active heterotetramer containing two p18 and two p10 subunits (28). This active heterotetramer then can then cleave effector caspases such as caspase 3, which in turn cleaves cellular products, resulting in apoptosis. Studies in FADD and caspase 8 deficient mice indicated that both are required for FAS-mediated apoptosis (29, 30).

Cells can be divided into two types according to their requirement for mitochondrial pathway in Fas-induced apoptosis. In type I cells, processed caspase 8 is sufficient to directly activate other members of the caspase family, and induce apoptosis. In type II cells, efficient activation of effector caspases by Fas depends on an amplification loop that relies on caspase 8-mediated cleavage of Bid and following release of mitochondrial proapoptotic factors such as SMAC/Diablo or cytochrome c to drive the formation of the caspase 9-activating apoptosome (31), active caspase 9 in turn activates the effector caspase 3 (32).

Another DED-containing protein, caspase 10, has also been shown to be recruited to the DISC (33). Although in vitro studies showed that caspase 10 shares similar features with caspase 8 in many aspects, in vivo studies demonstrated that it can not substitute for caspase 8 in the DISC to trigger apoptosis (34). The reason why caspase 10 is associated with the DISC while not being essential for death signaling is unknown.

FLICE-like inhibitory protein (FLIP) has also been identified in the DISC. After, two forms of FLIP were cloned (35, 36), termed c-FLIPS (short) and c-FLIPL (long). C-
FLIPS contains tandem DEDs and is highly homologous to the N-terminus of caspase 8. c-FLIPL also contains the tandem DEDs and a protease-like domain that is homologous to caspase 8 although lacking enzymatic activity. The role of FLIP in death receptor-mediated apoptosis is still controversial. FLIP can be recruited to the DISC of death receptors, thereby disabling DISC-mediated processing and release of active caspase 8, making FLIP an anti-apoptotic protein (37). However, transfection of c-FLIPL at levels comparable to physiological levels enhances apoptosis by promoting procaspase 8 processing in the CD95 DISC, while a decrease of c-FLIPL expression results in inhibition of apoptosis, suggesting that c-FLIPL is a pro-apoptotic protein (38).

Our group has recently reported calmodulin (CaM), the intracellular Ca\(^{2+}\) mediator, is recruited into the DISC (39). CaM is a dumbbell shaped protein that acts as a major Ca\(^{2+}\) sensor and regulator by interacting with a variety of cellular proteins. The globular ends each contain a pair of Ca\(^{2+}\)-binding domains; the binding of Ca\(^{2+}\) to CaM exposes a hydrophobic surface that binds numerous target proteins. We have shown direct binding between CaM and Fas that is regulated during Fas-induced apoptosis (40). The CaM-Fas binding was confirmed by several methods including immunoprecipitation and in vitro binding assays with purified proteins. In addition, CaM binds with c-FLIPL but not with FADD or caspase 8 (41). The interaction between CaM and c-FLIPL is furthermore to be Ca\(^{2+}\) dependent and regulated in response to Fas-activation.

**Intrinsic Pathway**

The intrinsic pathway is activated through several stimuli, which include DNA damage, cytoskeletal damage, endoplasmic reticulum stress, loss of adhesion, and growth
factor withdrawal (42). The intrinsic pathway, or mitochondrial pathway, is defined by mitochondrial outer membrane permeabilization. This process leads to the release of proteins, such as cytochrome c and apoptosis-inducing factor (AIF), that are normally located in the space between the inner and outer mitochondrial membrane (43). The outer membrane permeabilization can cause apoptosis through several different mechanisms including the release of molecules involved in the activation of caspases, the release of molecules involved in caspase-independent cell death, and also the loss of mitochondrial functions that are essential for cell survival.

The release of cytochrome c allows the formation of the apoptosome which in turn activates executioner caspases which carry out apoptosis. The components of the apoptosome are cytochrome c, an adapter molecule Apaf-1 (Apoptotic protease-activating factor) and pro-caspase 9. Apaf-1 binds to ATP or dATP and hydrolyzes to ADP or dADP, respectively; this complex can then bind to cytochrome c which promotes the multimerization of Apaf-1-cytochrome c complex. Finally, once the complex is formed, pro-caspase 9 is recruited to the complex, and becomes activated through autocatalysis. The activated caspase 9 is then released from the complex, allowing it to cleave downstream caspases, while new pro-caspase 9 are able to enter the complex to be cleaved (44). Apaf-1 binds to cytochrome c in the absence of dATP but does not form the oligomeric complex (45). The mitochondrial pathway may also operate through caspase-independent mechanisms as well, such as the release of mitochondrial proteins like apoptosis-inducing factor (AIF) and Endonuclease G which trigger DNA fragmentation without the help of caspases (46).
The occurrence of mitochondrial outer membrane permeabilization is highly regulated by proteins in the Bcl-2 family. Bax and Bak promote the permeabilization by forming openings (pores) in the outer mitochondrial membrane. They reside in the cell in their inactive forms, being activated by other pro-apoptotic Bcl-2 proteins. This process is inhibited by antiapoptotic Bcl-2 proteins that sequester the activating proteins and the active Bax or Bak proteins (47). The relative amounts of pro- and anti-apoptotic Bcl-2 family proteins determine the apoptotic sensitivity of cells to various stimuli and have been reported to correlate with various pathologies (48-50).

There is cross talk between extrinsic and intrinsic pathways through a Bcl-2 family member, Bid. Bid resides exclusively in the cytosol of living cells. Upon activation of cell surface receptors, Bid is cleaved by caspase 8; the truncated Bid (tBid) translocates from the cytosol to the mitochondria and induces the release of cytochrome c (33, 51).

Gemcitabine

Gemcitabine (2’,2’-difluorodeoxycytidine) is a deoxycytidine analogue. Gemcitabine (GMC) is hydrophilic and therefore needs a transporter to cross the plasma membrane, to exert its effects (52). Most of gemcitabine uptake is carried out through Hent1, a human nucleoside transporter. This is demonstrated by the fact that Hent1/−-cells are resistant to gemcitabine treatment (53). More compelling evidence is seen in the clinical setting, where the sensitivity to gemcitabine is correlated with the expression of this transporter (54, 55).
Once gemcitabine is inside the cell, it is phosphorylated to gemcitabine monophosphate by deoxycytidine kinase (Dck). This product is then converted to its active diphosphate (dFdCDP) and triphosphate (dFdCTP) metabolites. Phosphorylation of gemcitabine by Dck is the rate-limiting step for further phosphorylation to active metabolites and is essential for its cytotoxic activity (56). The active metabolites of GMC have several actions within the cell. The main mechanism of action of GMC is as a potent inhibitor of DNA synthesis, which occurs from the competition between dFdCTP and the normal DNA component dCTP (57, 58). Also, dFdCDP inhibits ribonucleotide reductase (RR) which depletes the cellular concentrations of dCTP, this action gives dFdCTP a higher chance of being incorporated into the DNA. Normally when dCTP is produced it negatively feeds back on Dck. If gemcitabine decreases the levels of dCTP Dck remains fully active allowing more of the gemcitabine to be phosphorylated. This kind of mechanism is termed self potentiation, where the drug acts in many ways to potentiate its cytotoxicity (60). An additional mechanism of action of dFdCTP is to inhibit the deoxycytidine monophosphate deaminase, which is also the same enzyme that deactivates gemcitabine. This inhibition allows the prolonged stability of the gemcitabine triphosphate and also an example of self potentiation (60).

GMC is currently used to treat cholangiocarcinoma (61) along with many other malignancies (52). It is still unclear by which downstream molecular pathway gemcitabine incorporation into DNA leads to cell death. Apoptosis-regulating genes, such as p53, bcl-2, bcl-XL, Bax regulate cancer cell sensitivity or resistance to gemcitabine, suggesting that the intrinsic pathway may be the way it causes apoptosis (62, 63). On the other hand, in some tumor cell lines gemcitabine induces apoptosis
through the activation of caspases, for example caspase 8 and 3, suggesting that gemcitabine may work through the extrinsic, or death receptor mediated pathway (64, 65).

**Tamoxifen**

Tamoxifen (TMX) is a nonsteroidal antiestrogen that has been used to treat breast cancer since the 70s. It is a lipophilic molecule so it readily passes through the plasma membrane. Once inside the cell, TMX is metabolized to three potent antiestrogens 4-hydroxytamoxifen, N-desmethyltamoxifen, and 4-hydroxy N-desmethyltamoxifen (endoxifen) (66). Each of these metabolites has similar or enhanced affinity for the estrogen receptor compared with the parent compound. TMX is a partial agonist of Estrogen Receptor-alpha, and a pure antagonist of Estrogen Receptor-beta (67).

Aside from its effects on the estrogen receptor (ER), TMX has also been shown to have ER-independent mechanisms for inducing apoptosis in human cancer cells, as illustrated from TMX’s effects on ER-negative cancers (68, 69). ER-independent pathways that have been reported for tamoxifen-induced apoptosis are protein kinase C, CaM, c-myc, MAPK, TGF-beta and AKT (68).

McDonald’s group has been interested in the CaM-dependent effects of TMX in cholangiocarcinoma cells that lack the estrogen receptor (70). TMX, along with other CaM-antagonists, induce apoptosis of these cells in a Fas-related manner (11, 39, 70). CaM has been found to bind to Fas (40), c-FLIPL (41) and be recruited to the Fas-activated DISC upon Fas stimulation (39). CaM binds to Fas through a classical Ca^{2+}-dependent 1-5-10 motif (40). This interaction is specific because CaM does not bind to
other members of the TNF family, DR4, DR5, and TRAIL. Analysis of the Fas-activated DISC demonstrate that CaM binds to FLIP\textsubscript{L} but not FADD or caspase 8. The interaction between CaM and c-FLIP\textsubscript{L} is also specific because CaM does not bind with c-FLIP\textsubscript{S}. CaM binds to FLIP\textsubscript{L} in a Ca\textsuperscript{2+}-dependent manner, however, the two classical Ca\textsuperscript{2+}-dependent CaM binding motifs, 1-5-10 and 1-8-14, were not observed in this interaction (41). The disruption of CaM-FLIP binding resulted in cells that are more sensitive to Fas-mediated apoptosis. The fact that both of these interactions are regulated by Fas stimulation and they appear to bind through different motifs, this could potentially be used to develop specific inhibitors of one of these CaM interactions.
MATERIALS AND METHODS

Cell Culture, Antibodies and Reagents

The cholangiocarcinoma cell line, Sk-Cha-1, was kindly provided by Dr. A. Knuth (Ludwig Institute for Cancer Research, London, UK). Cells were grown in RPMI 1640 medium (Invitrogen) supplemented with penicillin (5U/ml), streptomycin (5µg/ml), and 10% heat-inactivated fetal bovine serum. Antibodies include: anti-Fas (Santa Cruz Biotech, CA), anti-GAPDH (Research Diag. Inc. Concord, MA) and anti-cytochrome c (BD Pharmingen, San Jose, CA). Goat anti-mouse and bovine anti-rabbit HRP conjugated antibodies (Santa Cruz Biotch, CA). Tamoxifen Citrate (Calbiochem, La Jolla, CA) was dissolved in 100% ethanol at a concentration of 10mM. Before each use the TMX was re-dissolved over heat. Gemcitabine HCl (Eli Lilly, Indianapolis, IN) was dissolved in phosphate buffered saline (PBS) at a concentration of 10 mM.

Assessment of Apoptosis

Annexin V-fluorescein isothiocyanate and propidium iodide (PI) staining was performed using an apoptosis detection kit (BD Biosciences, Palo Alto, CA) and analyzed by flow cytometry according to the manufacturer’s recommendations. The percentage of cells that were solely Annexin V positive and Annexin V and PI positive were measured apoptotic. For Tamoxifen and Gemcitabine dose curves, 6x10^5 cells were seeded into 6-well plates and exposed to doses ranges of 1-10 µM Gemcitabine or 5-30 µM Tamoxifen for 24 hours. For Tamoxifen and Gemcitabine combination studies, 6x10^5
cells were seeded into 6-well plates and exposed to 15 μM Tamoxifen and/or 5 μM Gemcitabine.

Western Blot Analysis

For Western Blot analysis of Fas and GaPDH, whole cell protein lysates were isolated from cells using lysis buffer containing 100mM Tris-HCl, pH 8.0, 150mM NaCl, 1% sodium dodecyl sulfate, 10% glycerol, 1% NP-40, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1mM sodium orthovanadate, 1mM β-glycerophosphate and protease inhibitor cocktail tablets (Roche, Indianapolis, IN).

In separate studies, we determined cytochrome c release by cytosolic extracts which were prepared using a lysis buffer containing 20mM HEPES-KOH, pH 7.5, 10mM KCl, 1.5mM MgCl2, 1mM EGTA, 1mM DTT, 250mM sucrose and protease inhibitors. Cell solutions were homogenized using a dounce homogenizer and centrifuged at 1,000xg for 10 minutes then the supernatant was removed and centrifuged at 16,000xg for 10 minutes. The remaining supernatant was collected as the cytosolic fraction.

Concentrations of proteins were determined with the Bicinchoninic acid kit (Sigma). Proteins were separated by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% milk, and incubated with primary antibodies overnight at 4°C. After 16 hours of incubation with the primary antibody, membranes were washed three times with 1X tris buffered saline plus tween-20 (TBST) for 10 minutes each. Horseradish peroxidase (HRP) conjugated secondary antibodies in
the blocking buffer were incubated for 2 hours at room temperature. After the secondary antibody was removed, membranes were washed again using 1x TBST for 10 minutes, three times. Signals were detected using immobilon chemiluminescent HRP-substrate (Millipore, Billerica, MA) detection kit.

Cell Cycle Analysis

Cells were plated at 70-80% confluence and treated with 15 μM Tamoxifen or 5 μM Gemcitabine for 16 hours. Cells were trypsinized, centrifuged and washed with PBS 2X, PBS was removed and the cells were fixed by adding 0.5mL 0.9% NaCl to the tube while gently vortexing. 4°C 95% ethanol was added drop-wise to this solution (1.5mL) and the cells were left on a rotator at 4°C overnight. The cells were centrifuged and the supernatant was removed and 150μL of 1mg/mL of RNAse in PBS was added to the solution. The cells were transferred to 12x75mm culture tubes and incubated in the 37°C water bath for 20 minutes. Right before taking the cells for flow cytometry analysis 150 μL of 40 μg/mL of propidium iodide in PBS was added to the cells.

Caspase Activity Assays

Caspase-3, -8,-9, -10 kits were from BioVision (Mountain View, CA) and instructions were followed as written. 1.6x10^6 cells were plated into 60 mm dishes. The cells were treated for 16 hours with 20 μM Tamoxifen or 5 μM Gemcitabine, and lysed using the cell lysis buffer provided within the kit, for 10 minutes on ice. The cells were centrifuged at 10,000 rpm for 10 minutes, the supernatant was removed and protein concentration was evaluated using the BCA protein kit. 100 μg of protein was used for
each sample was loaded into a 96-well plate. The 100 μg protein was diluted to 50 μL with cell lysis buffer. 50 μL of 2X Reaction Buffer (supplied with the kit) with 10 mM DTT was added to the protein. Added to the total of 100 μL was 5 μL of the provided substrate. The 96-well plate was left to incubate for 1.5 hours, after which the plate was read at an absorbance of 400nm. A blank absorbance obtained from a well containing all reagents except for protein was subtracted from each of the 400nm sample absorbencies. These numbers were then normalized to the control, untreated, sample of each reaction.

Mouse Xenograft Model

The animal use protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham, AL, USA. Eight-week old athymic (nu/nu) male Balb/c mice (Charles Rivers Laboratories, MA) were used for tumor inoculation. Briefly, cholangiocarcinoma cells (5 x 10^6 cells in 200 μL PBS/site) were inoculated subcutaneously into the flanks of mice. Tamoxifen (TMX) was purchased from Calbiochem, CA. After the tumor size reached 60-70 mm^3, mice were randomly assigned into four groups. Control group was treated with vehicle, Gemcitabine alone group was treated with 120mg/kg ip every 3 days, Tamoxifen alone group was treated with 15mg/kg ip for 2 consecutive days with one day of rest, and finally a combination group was treated with 15mg/kg TMX ip for 2 consecutive days followed by one day of rest and 120mg/kg GMC ip every 3 days. The tumor volumes and weights of the animals were measured every three days over the length of the study (4 weeks). Tumor volume was calculated using the formula Volume= Length x Width^2/2. At the time of sacrifice, blood and tumors were collected for further analysis.
RESULTS

Effects of Gemcitabine and Tamoxifen on apoptosis of cholangiocarcinoma cells

Previous studies have demonstrated that Cholangiocarcinoma expressed heterogeneous Fas Receptor expression. Therefore we confirmed the expression of Fas in the two cell lines by Western Blot (Figure 1A). Western Blot of the cell line expression less Fas (Fas L) is shown in the left lane, and the line expressing higher Fas (Fas H) is shown in the right lane. To determine whether Gemcitabine (GMC) and Tamoxifen (TMX) affect apoptosis, Fas H and Fas L cholangiocarcinoma cells were treated with 5 μM-20 μM and 1 μM-10 μM of TMX and GMC, respectively for 24 hours. As seen in Figure 1B, GMC induced apoptosis of both Fas-High and Fas-Low cells in a dose-dependent manner, with a maximum apoptosis of 43.2 ± 2.7% and 45.6 ± 6.8%, respectively, occurring at 10 μM (n=3, mean ± SE). By contrast, TMX induced apoptosis only in Fas L cells (Figure 1C). Fas High cells treated with TMX responded in a dose-dependent manner with the maximum dose apoptosing 44.8 ± 4.1% of the cells, whereas the Fas Low cells treated with the same doses elicited only 18.9 ± 3.0% apoptosis (n=3, mean ± SE). Therefore, TMX appeared to exert its effects in a Fas-dependent manner, while GMC acted through a mechanism that is Fas-independent. Accordingly, TMX and GMC might have an additive or synergistic effect on apoptosis when used in combination.

To test whether the cells responded to the combination therapy in an additive or synergistic manner, the cells were exposed to TMX alone, GMC alone or TMX and GMC
for 24 hours. As shown in Figure 2, combination therapy additively induces apoptosis, with 23.8 ± 0.8% apoptotic cells for 15 μM TMX alone, 31.5 ± 2.8% apoptotic cells for 5 μM GMC alone, or 47.2 ± 3.9% for the combination of the drugs at those concentrations (n=3, mean ± SE).

Actions of GMC and TMX within the cell

*Effects of GMC and TMX on cell cycle*

GMC has been shown to cause cell cycle arrest in lung cancer, pancreatic cancer and hepatocellular cancer cell lines (62, 71, 72). Therefore, we analyzed the effects of GMC and TMX on cell cycle. Treatment of cells with 5 μM GMC caused an increase in the percentage of cells in the G1 phase, while TMX at 15 μM had no effect on cell cycle (Figure 3). This is demonstrated by 84.8 ± 0.01% cells in the G1 phase after treatment with GMC compared with either 54.8 ± 3.6% or 60.4 ± 2.04% for the control or TMX treated samples, respectively (n=3, mean ± SE, p<0.001).

*Effects of GMC and TMX on caspase activation and cytochrome c release*

Our previous work has shown that TMX activates caspase 8 and 10 consistent with it acting through the Fas or receptor-mediated pathway (11). To analyze differences in the activation of caspases involved in the actions of TMX and GMC, caspase activity assays were performed as described in the methods. As shown in Figure 4, TMX activated extrinsic-associated caspases 8 and 10, while GMC treatment activated caspases 8, 9 and 10. Furthermore, cytosolic extracts were prepared from cells treated for 8 hours with TMX and GMC, and Western Blots for cytochrome c performed. Gemcitabine
increased cytosolic cytochrome c as compared with control or TMX treated cells, consistent with GMC but not TMX activating the mitochondrial-activated apoptotic pathway (73).

Effects of GMC and TMX on tumor growth in mouse xenograft model

To determine effects of TMX and GMC therapy on tumor growth in vivo we utilized a xenograft model. Dr. Gu Jing Ph.D, a Research Associate in the McDonald lab performed the injections and final analysis, but I assisted in day-to-day management of animals, such as weighing, measuring tumor volume, and pre-experiment planning of preparing the protocol. The study was carried out as described in the methods. GMC, TMX and the combination inhibited tumor growth compared with the control in this in vivo model (Figure 6A-B).
FIGURES

A. Fas L    Fas H


Fas (60 kDa)

GAPDH (37 kDa)

B. GMC

% apoptotic cells

Fas High
Fas Low

control 1uM 3uM 5uM 10uM
Figure 1. The effects of Tamoxifen and Gemcitabine on apoptosis of Cholangiocarcinoma cells expressing high and low levels of Fas.

A. Western Blot analysis of untreated cholangiocarcinoma cell lysates probed for Fas. B. Fas High and Low cholangiocarcinoma cells were treated with various doses of GMC as shown in the figure for 24 hours. C. Fas High and Low cholangiocarcinoma cells were treated with various doses of TMX as shown in the figure for 24 hours. The apoptotic death was determined by staining the cells with Annexin V/Propidium iodide (PI) and counting Annexin V positive and PI negative cells. The data represents mean ± SE (n=3, * p<0.05, **p<0.01). Apoptotic death was determined by staining the cells with Annexin V/Propidium iodide (PI) and counting Annexin V positive alone and Annexin V and PI positive cells. The data represents mean ± SE (n=3).
Figure 2. Additive effects of Tamoxifen + Gemcitabine on Cholangiocarcinoma cells
Cholangiocarcinoma cells were treated with TMX, GMC or TMX and GMC at the concentrations shown for 24 hours and apoptotic cell death was analyzed. The data represent mean ± SE (n=3, * p<0.05, **p<0.01).
Cholangiocarcinoma cells were treated with TMX or GMC for 16 hours. Cell cycle analysis was performed. GMC treated cells showed an increase in the percentage of G1 phase cells, indicating a cell cycle arrest, while TMX had no effect on cell cycle. The data represent mean ± SE (n=3, **p<0.01).
Figure 4. Effect of Tamoxifen and Gemcitabine on caspase activity
Cholangiocarcinoma cells were treated with 20 μM TMX or 5 μM GMC for 16 hours. Caspase activity was measured using kits purchased from BioVison, where the amount of cleaved substrate correlates with caspase activity of each sample. The cleaved substrate is seen as a color change that can be read at an absorbance of 400 nm. A blank was subtracted from the raw absorbances and for each caspase the samples were normalized to the control. Tamoxifen activates caspases 8, 10 and 3, whereas Gemcitabine activates caspases 8, 9, 10 and 3. The data represents average ± SE (n=3, *p<0.05).
Figure 5. Gemcitabine activates the release of cytochrome c from the mitochondria. Cholangiocarcinoma cells were treated with 15 μM TMX (T) or 5 μM Gemcitabine (G) for 8 hours. Cytosolic lysates were analyzed for cytochrome c expression by Western Blot. GMC increases the level of cytochrome c in the cytosol after 8 hours, while TMX and the control (C) cells do not. This data represents three independent experiments.
Amy Turk assisting Gu Jing PhD for in vivo experiments
Figure 6. Gemcitabine, Tamoxifen and the combination inhibit Cholangiocarcinoma tumor growth.
Cholangiocarcinoma cells were injected into the flanks of 8 week old athymic nude male mice using the protocol outlined previously (11). The tumor volumes and weights of the animals were measured every three days over the length of the study (4 weeks). After tumors reached a volume of 60-70 mm$^3$, the mice were randomly assigned into 4 groups; Control treated with vehicle, Gemcitabine treated with 120mg/kg ip every 3 days, Tamoxifen treated with 15mg/kg ip for 2 consecutive days with one day of rest, and finally a combination group that was treated with 15mg/kg TMX ip for 2 consecutive days followed by one day of rest and 120mg/kg GMC ip every 3 days. At the time of sacrifice, blood and tumors were collected for further analysis. A. Gemcitabine, Tamoxifen and their combination inhibited tumor growth. B. Representative tumors from each group that were removed from the mice after sacrifice.
Figure 7. Proposed model for Tamoxifen and Gemcitabine-induced apoptosis

Tamoxifen induces apoptosis through the activation of the extrinsic pathway, shown by the activation of caspases 8 and 10. Gemcitabine causes DNA damage that initially results in cell cycle arrest and then apoptosis through the mitochondrial or intrinsic pathway, shown by the release of cytochrome c and activation of caspase 9. The arrow pointing from GMC to caspase 8/10 indicates a possible direct cleavage of these caspases by GMC.
DISCUSSION

Cholangiocarcinoma is a highly lethal tumor arising from the biliary epithelium that has a very poor 5-year survival rate due to the lack of early diagnosis and effective therapies. Gemcitabine is a drug that is currently used for patients with this disease; it is a nucleoside analog that inhibits DNA synthesis, arrests the cell cycle (Figure 3), and activates mitochondrial apoptotic pathway (Figures 4 & 5). Tamoxifen is a widely used drug that has low side effects even at high doses, and is inexpensive. It has been shown that Tamoxifen acts as a CaM antagonist in these Estrogen Receptor negative Cholangiocarcinoma cells (70). Treatment of cholangiocarcinoma cells with TMX decreases the levels of phosphorylated AKT and c-FLIPL proteins (11). McDonald’s group has previously shown that Tamoxifen induces apoptosis of cholangiocarcinoma cells, both in vitro and in vivo in a mouse xenograft model (11, 70). They have shown that other CaM antagonists, such as Trifluoperazine and W-7, also induced apoptosis in human cholangiocarcinoma cells. In addition, the apoptosis-inducing ability of TMX was found to be Fas-dependent, shown by the inability of TMX to induce apoptosis in cells with very low levels of Fas, as compared with cells with that express a higher amount of Fas (Figure 1C).

We propose that the ability of Tamoxifen and Gemcitabine to produce an additive effect is due to differing mechanisms of each agent within the cell. We have previously shown that TMX induces apoptosis in cholangiocarcinoma cells in a Fas-
related manner (70). As shown in Figure 7, TMX inhibits the recruitment of CaM to the Fas-activated DISC, which may lead to the decrease of survival signals sent through the CaM-FLIP<sub>L</sub> interaction (unpublished preliminary results from our lab). Through work done in this thesis, it appears that GMC exerts its apoptotic effects primarily through the intrinsic mitochondrial apoptotic pathway. GMC causes the release of cytochrome c from the mitochondria after 8 hours (Figure 5) and activates caspase 9 after 16 hours (Figure 4B). The release of cytochrome c results in the formation of the apoptosome which then leads to the cleavage of caspase 9 (44). The results also indicate that after 16 hours, GMC minimally, as compared with TMX, cleaves caspase 8 and 10 (Figure 4A & C). GMC may be directly activating the cleavage of caspase 8 and 10, to test this hypothesis, caspase 8 and 10 inhibitors could be used as a pre-treatment to see if the apoptotic effects of GMC are altered.

These in vitro and preliminary in vivo studies are part of a multidisciplinary research program that includes crystallographers and clinicians. The combination of Tamoxifen and Gemcitabine was shown to produce an additive apoptotic effect in vitro on human cholangiocarcinoma cells (Figure 2). These positive results led us to test the same hypothesis in the intact animal (Figure 6). Tamoxifen alone and Gemcitabine alone effectively inhibited tumor growth in the cholangiocarcinoma xenograft animal model. The effects of the combination therapy are unclear at this time due to the large responses of TMX and GMC alone. We have ongoing experiments to adjust the protocol, including lowering the doses of Gemcitabine given to the animals and also trying to give the agents sequentially with a pre-treatment of Tamoxifen, followed with . Gemcitabine is a drug that has many side effects and if given with Tamoxifen the doses may be reduced,
allowing a treatment plan with fewer side effects. Positive results in this area could potentially lead to a clinical trial.

CaM is a docking protein within the Fas-activated DISC that may integrate signals of survival and death (74). CaM binds with both Fas and FLIP in Ca$^{2+}$ dependent manners, however, their CaM-binding sites are structurally different. CaM binds with Fas through a classic 1-5-10 motif (40), the binding with FLIP is mediated via a non-classical motif (41). This indicates that there may be differences in the binding of CaM to each of these sites, and that targeting one of them specifically could provide a basis for rationale drug design. The Fas intracellular domain has been crystallized (75), but FLIP has not. A collaborator, Dr, Yuhua Song, is modeling the binding between Fas and CaM and has found that there is a conformation change that takes place with both CaM and Fas after their binding (76). A Research Associate in the McDonald lab, Dr. Gu Jing, is currently purifying wild-type FLIP and mutant FLIP proteins that are void of the CaM binding domain to crystallize them. The mutant proteins do not bind to CaM. Understanding the relationship between CaM, Fas and FLIP in the DISC is a prerequisite for designing novel targeted anticancer therapy.
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