2008

Calmodulin Binding To Cellular Flice Like Inhibitory Protein Modulates Fas-Induced Signaling And Tumorigenesis In Cholangiocarcinoma

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2008

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GRADUATE PROGRAM: PATHOLOGY

ABSTRACT

Cholangiocarcinoma, a fatal tumor arising from biliary epithelium, has very poor 5-year survival rate due to lack of early diagnosis and effective therapies. Induction of the Fas-mediated apoptosis is a promising therapeutic target in this tumor.

Studies from our group and others have indicated that Fas-expression correlates inversely with disease progression and that CaM-antagonists induce apoptosis in cholangiocarcinoma cells in a Fas related manner. Further, we reported a direct and dynamic interaction of CaM and Fas and that CaM is recruited into the Fas-stimulated death inducing signaling complex (DISC), suggesting a cross talk between Fas and CaM pathways. The studies presented in this dissertation were designed to characterize the role of CaM in Fas-induced signaling and identify potential therapies for cholangiocarcinoma.

Fas-mediated apoptosis typically involves the recruitment of an adapter protein, FADD, caspase-8 and/or c-FLIP to form the DISC. Screening for the CaM binding proteins in the DISC, we demonstrated a Ca^{++}-dependent direct interaction between CaM and $FLIP_L$, but not the other DISC components, FADD, caspase-8 and $FLIP_S$. Fas activation induced a Ca++ dependent increase in CaM-FLIP binding which was inhibited by CaM-antagonist, trifluoperazine (TFP), with concurrent inhibition of ERK

phosphorylation and increased FLIP binding with ubiquitin. The CaM binding region was identified between aa 197-213 on $FLIP_L$.

Over expression of $FLIP_L$ decreased the sensitivity of cholangiocarcinoma cells to Fas and CaM-antagonist-induced apoptosis and increased their tumorigenicity in nude mice. Deletion of the CaM binding region from $FLIP_L$ restored the sensitivity of cholangiocarcinoma cells to Fas and CaM-antagonist-induced apoptosis and decreased their tumorigenicity in nude mice.

The CaM-antagonist, tamoxifen (TMX), induced apoptosis in caspase dependent and independent manners with concurrent inhibition of pAKT and FLIP expression in cholangiocarcinoma cells. A combination of TMX and Gemcitabine, a currently used therapy for cholangiocarcinoma, induced more apoptosis than treatment with either agent alone.

Thus the results of this dissertation support the concept that the CaM-FLIP binding is an important regulator of Fas- and CaM-antagonist-induced apoptosis and that it may provide a novel therapeutic target for cholangiocarcinoma. Further the CaM-antagonist, TMX, may be used alone or in combination with Gemcitabine or other antineoplastic compounds as a therapy for cholangiocarcinoma.

TABLE OF CONTENTS

LIST OF FIGURES

Figure *Page*

CALMODULIN BINDING TO CELLULAR FLICE LIKE INHIBITORY PROTEIN MODULATES FAS INDUCED SIGNALING

MOLECULAR MECHANISMS OF TAMOXIFEN THERAPY FOR CHOLANGIOCARCINOMA: ROLE OF CALMODULIN AND APOPTOSIS

LIST OF FIGURES (Continued)

Figure *Page*

UNPUBLISHED DATA

CONCLUSION

INTRODUCTION

Apoptosis

Apoptosis is a tightly regulated, genetically programmed and energy dependent process by which abnormal cells are removed from the body without generating an inflammatory response [1]. The process is crucial not only during embryonic development for proper organogenesis but also throughout the life to maintain cellular homeostasis [2, 3] . Under physiologic conditions apoptotic cell death could occur in situations when the cells have fulfilled their functions and are no longer required, have failed to develop normally, or have accumulated DNA damage that is irreparable [4, 5]. Thus a homeostatic balance is maintained between healthy cells undergoing proliferation and senescent cells undergoing death. Too much or too little apoptosis disturbs this homeostasis, leading to various pathological conditions such as autoimmunity, neurodegeneration and cancer [6].

Cells undergoing apoptotic death exhibit features such as cell shrinkage, chromatin condensation, nuclear fragmentation, cytoplasmic blebbing and fragmentation into small apoptotic bodies which are then removed by neighboring cells or macrophages [4, 7]. As there is no release of any cellular contents into the intercellular environment, an inflammatory response is not generated. On the other hand in necrosis, cells undergo swelling, organelle fragmentation and release of cellular contents, thus initiating an inflammatory response. Apoptosis is referred to as programmed cell death which can be

initiated by various triggers such as cell surface death receptor ligands [8, 9] chemotherapeutic agents [10], infections [11] and stress such as ionizing radiation [12] or ultraviolet light [13] . Cell death can also be initiated by removal of death inhibiting or pro-survival ligands which allow endogenous death machinery to proceed unchecked [14-16].

Whatever the trigger is that initiates apoptotic cell death, the basic machinery used and the resulting cellular morphology remains similar for the most part. The hallmark of this process is the initiation of a cascade of reactions that involves activation (induced by cleavage) of specific proteases called caspases. The cleaved initiator caspases in turn activate downstream effector caspases leading to activation of various substrates that lead to DNA fragmentation and ultimately cell death. Interestingly the current understanding of the genetics and molecular mechanisms behind apoptotic cell death emanated from the studies with the nematode, Caenorhabditis elegans. Programmed cell death is so specific in C. elegans that out of 1090 somatic cells formed during development of an adult hermaphrodite; exactly 131 cells undergo apoptotic death leaving an adult with 959 cells. Further, these apoptotic events occur in every animal and at their own characteristic times indicating how tightly the process is regulated [17]. Further, genetic analysis of the process identified that genes ced-3 and ced-4 in C. elegans are required for apoptosis and the ced-9 gene is an inhibitor of apoptosis [18]. This model of programmed cell death in C. elegans is conserved throughout evolution and hence is relevant in higher animals as well, though with added complexity [19]. Likewise in humans, the apoptotic process is controlled by various mediators that either help initiate and propagate the process or interfere with it. The relative expressions of

these pro- and anti-apoptotic signaling molecules decide the fate of the cell in response to various stimuli.

Over the years, dysregulation of apoptosis has gained considerable attention not only in the basic sciences but also in clinical medicine [19]. As an example, the discovery of the bcl-2 gene, which encodes an anti-apoptotic protein, emphasizes the importance of failure of apoptosis in causing cancer and the potential of such molecules as therapeutic targets [20]. Interestingly, the importance of induction of apoptosis as a potential tool to kill cancer cells was reported as early as 1975 [21]. Thus, alterations in the apoptotic machinery have been known to contribute to the initiation and progression of a variety of diseases including malignancies and also potentially form the basis of therapies [22, 23] [24]. Characterizing the molecular mechanisms involved in dysregulation of apoptotic pathways has been an important facet of cancer research and has helped identify likely molecular targets that could potentially be exploited therapeutically.

Pathways of apoptosis

Simplistically, the apoptotic cell death can be initiated through two pathways as shown in Fig.1: an extrinsic pathway operating through cell surface receptors called death receptors and an intrinsic pathway which engages the mitochondrial machinery [25].

Extrinsic pathway

The extrinsic pathway proceeds through cell surface receptors called death receptors which belong to a Tumor Necrosis Factor (TNF) super family of receptors. These are type I transmembrane proteins sharing two to four homologous cysteine rich

extracellular domains and there are eight such receptors described in this family so far [26]. The cytoplasmic regions of these receptors are generally not related with the exception of two receptors, Fas (CD95/ApoI) and TNF- receptor-1 (TNF-R1), which share a homologous cytoplasmic region called the death domain (DD) [27, 28]. In response to their cognate ligands, these receptors undergo a conformational change allowing them to interact, via their death domains, with adapter proteins, such as FADD or TRADD, also containing homologous DD regions [29]. The adapter protein recruits initiator caspases such as caspase-8 and/or caspase-10 through the interaction between another homologous domain termed death effector domain (DED) to form the death inducing signaling complex (DISC) thus initiating a cascade of caspase cleavage [30-34].

Fas (CD95/APO-1), a 45-kDa transmembrane protein, is an extensively studied and well characterized member of the TNF receptor super family and was first described as a death receptor [35]. It is expressed in many cell types as a cell surface receptor [36]. The interactions between extracellular cysteine rich domains, also called preligand binding assembly domains (PLAD), lead to the formation of preassociated homotrimers on the cell surface [37, 38]. The preassociation is important in signal transduction through the Fas receptor as mutations in this domain have been reported to result in autoimmune lymphoproliferative syndrome (ALPS) [38]. Thus it is believed that clustering of Fas is required for efficient signaling [39]. The cytoplasmic tail of Fas contains a conserved protein-protein interaction domain called the death domain (DD) which serves as a platform for the recruitment of homologous proteins leading to formation of the DISC [40]. Unlike the Fas receptor which is expressed ubiquitously in numerous tissues, the expression pattern of its cognate ligand FasL is restricted and

tightly regulated. Thus FasL expressed on activated T lymphocytes is important in eliminating transformed or infected cells [41]. With Fas-FasL interaction, the preassociated Fas receptor undergoes a conformational change that allows its DD to interact with the DD of an adapter protein Fas-associated death domain containing protein (FADD) [30, 42]. FADD through homotypic DED interactions further recruits other DED containing proteins such as caspase-8, -10 or cellular FLICE like inhibitory protein (c-FLIP) to form the DISC [43]. The caspases undergo cleavage and subsequent activation within the DISC to set off a cascade of reactions that activate several downstream caspases, such as caspase-3, ultimately resulting in apoptotic cell death.

Although Fas has been described conventionally as a death receptor, recent studies using several knock out models suggest that the Fas receptor and the proteins integral to Fas signaling such as FADD and caspase-8 also play important roles in sending survival signals using the same machinery. Thus FADD and caspase-8 knock out mice are embryonic lethal [44, 45]. Several in vitro studies corroborated these reports, suggesting that the same proteins involved in Fas-induced death pathway play critical roles in cell survival/proliferation as well [46-50]. Thus molecular characterization of signaling pathways emanating from the same surface receptor such as Fas is important for better understanding of these distinct events and for identifying novel molecular targets that are crucial in switching between the death vs survival signals in response to the same ligand. One of the important molecular events that allows Fas receptor to act with such a diversity is the interaction of its cytoplasmic tail with different proteins that modulate the formation of the DISC, thus allowing it to send different signals. Thus in addition to FADD, proteins such as Fas associated phosphatase-1 (FAP-1), Daxx and

receptor interacting protein (RIP) have also been reported to interact with Fas cytoplasmic tail and modulate downstream signaling [51, 52]. Our group has recently established one such novel binding partner of Fas, calmodulin (CaM) [53]. A direct and dynamic binding was reported between CaM and the Fas DD consisting of a classical, Ca++ dependent 1-5-10 CaM binding motif. Further, CaM is recruited into the Fas-induced DISC [54] and CaM-antagonists induce apoptosis of cholangiocarcinoma cells in a Fas related manner [55, 56], suggesting that CaM may be involved in the regulation of Fas-mediated signaling.

Caspases

Caspases (Cysteine ASPartyl-specific proteASES) are the central executioners in apoptotic signaling that lead to the biochemical and morphological changes seen in apoptosis [57]. The most distinctive feature of this protease family is the ability to undergo maturation by cleavage on the carboxyl side of an aspartate residue (termed the P1 residue) [58, 59]. The presence of the aspartate residue at the P1 position is an absolute requirement for cleavage [60]. All caspases are present in an inactive (zymogen) form in the cells. After recruitment into the DISC, the initiator caspases form homodimers and get activated by autoproteolytic cleavage (proximity induced self processing model-caspases forming dimers cleave each other due to proximity) [42, 58, 59]. Typically there are two cleavage steps that generate active caspase heterotetramers containing a large and a small subunit that further activate or inhibit various downstream substrates resulting in apoptotic cell death [61]. Depending upon their structure and whether the caspases are involved in initiation or execution of the apoptotic program,

they are grouped into initiator and effector caspases. Thus initiator caspases such as caspase-8 and -10 but not the effector caspases have a long N-terminal prodomain. This prodomain consists of two types of interaction modules: a death effector domain (DED) found in caspase-8 and -10 or a caspase-associated recruitment domain (CARD) found in caspase-4 and-9 which interact with adapter proteins in the DISC [62, 63].

Effector caspases such as caspase-3 and -6 have short prodomains and do not contain DED or CARD [64]. These caspases operate downstream in the apoptotic machinery and cleave a wide variety of substrates such as poly (ADP-ribose) polymerase (PARP), DNA fragmentation factor (DFF), inhibitor of caspase activated DNAase (ICAD), nuclear envelope proteins and structural proteins that are responsible for the morphological changes seen during apoptosis [65-69].

c-FLIP (cellular FLICE like inhibitory protein)

c-FLIP has been described largely as an anti-apoptotic protein that interrupts death receptor mediated apoptotic signaling by interfering with recruitment and/or activation of initiator caspases into the DISC [70]. It was first identified as a viral protein v-FLIP, expressed by γ-herpesviruses that blocked Fas-mediated apoptosis [71, 72]. A bioinformatics search for a novel virus-encoded DED containing molecule regulating apoptotic signaling led to the discovery of v-FLIP. The mammalian homologue of v-FLIP is known by several names such as caspase homologue (CASH), Casper, caspase-8 and FADD-like apoptosis regulatory precursor (CLARP), inhibitor of FLICE (I-FLICE), FADD like anti-apoptotic molecule (FLAME), MACH-related inducer of toxicity (MRIT) and usurpin [73-78]. It shares structural and sequence homology with caspase-8

but lacks the proteolytic activity. It consists of two DEDs at its N-terminal and a C-terminal tail that shares sequence and structural homology with caspase-8 and-10. At the mRNA level, 11 distinct splice variants of c-FLIP have been reported [79]. At the protein level however, only two isoforms; a long isoform, $FLIP_L$ (55kDa), and a short isoform, $FLIP_S$ (28kDa), have been extensively studied. $FLIP_L$ shares structural homology with caspase-8, with two N-terminal DEDs and a long c-terminal tail homologous to caspase-8 but lacking protease activity due to the absence of cysteine residues in the catalytic subunit that are essential for caspase activity [73, 80]. Thus it is recruited into the DISC by FADD via homotypic DED interactions. Because of its structure, $FLIP_L$ has been reported to have dual functions in apoptotic signaling; it is believed to be inhibitory by interfering with recruitment and/or activation of caspase-8 in the DISC [81]. Some reports also describe its role in activation of caspase-8 by forming a heterodimer with it which helps achieve the initial cleavage step of caspase-8 [82, 83]. Thus when $FLIP_L$ is recruited into the DISC, it heterodimerizes with an initiator caspase. Due to the homology between the C-terminal tails of these two molecules, there is only a partial cleavage of caspase and $FLIP_L$ with no subsequent caspase cleavage steps due to lack of proteolytic activity in $FLIP_L$, resulting in a partially cleaved, inactive caspase molecule. However in some cell types the same mechanism allows $FLIP_L$ to achieve rapid initial processing of an initiator caspase and enhances apoptotic signaling when over expressed [78]. $FLIP_S$ on the other hand consists of two DEDs with a short c-terminal tail so that when it is recruited into the DISC it inhibits caspase cleavage altogether and is thus believed to be exclusively anti-apoptotic. Nevertheless, at comparable expression levels $FLIP_L$ is believed to be more potent inhibitor of apoptosis than $FLIP_S$ [70]. Current

dogma is that the expression of $FLIP_L$ could be a key determinant in deciding the fate of the cells in response to stressful stimuli [84, 85].

There is growing evidence supporting a role of c-FLIP in multiple signaling pathways. The phenotype displayed by FLIP deficient mice is similar to that of caspase-8 deficient mice and in both cases mice are embryonic lethal beyond days 10.5-12 [86]. This underscores the developmental importance of this protein and adds further complexity to its biological functions. Several recent studies have shown that c-FLIP not only interferes with Fas-induced apoptotic signaling, but also directs the signaling towards survival pathways. Thus c-FLIP has been linked with several non-apoptotic signaling pathways initiated through the Fas receptor such as activation of NF-kB and ERK [87-89]. Further, c-FLIP has been speculated to be an important docking protein to recruit various pro-survival molecules into the DISC and has been shown to interact with proteins such as TRAF1, TRAF2, Raf-1 and RIP to induce activation of NF-kB and ERK pathways. Therefore c-FLIP may provide a switch that allows death receptors such as Fas to transmit either death or survival signals, depending upon the expression levels of c-FLIP.

The role of the Fas receptor in pro-survival signaling was evident in FADD deficient mice that showed impaired T cell proliferation [90, 91]. Accordingly, Fas stimulation was shown to activate neuronal growth via activation of the ERK pathway [92]. Thus after the initial description of Fas as a prototype death receptor, extensive characterization of molecular components involved in this signaling pathway has led to a much broader functional implications of this receptor.

The intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis is operated through mitochondrial machinery which is activated by a variety of stimuli such as DNA damage, chemotherapeutic drugs and infections. These stresses induce changes in mitochondrial membrane potential resulting in formation of mitochondrial permeability transition pores. Formation of these pores release cytochrome-c from the mitochondrial inter-membrane space which then associates with Apaf-1 and caspase-9 to form the apoptosome that activates terminal caspases such as caspase-3, ultimately leading to cell death [93, 94]. The intrinsic pathway may operate through caspase independent mechanisms as well. Thus release of mitochondrial proteins like apoptosis-inducing factor (AIF) or EndoG triggers caspase-independent DNA fragmentation [95, 96].

There is cross talk between extrinsic and intrinsic apoptotic pathways via a Bcl-2 family member, Bid. Bid is cleaved by activated caspase-8 and translocation of truncated Bid to mitochondria induces changes in mitochondrial membrane potential, causing formation of permeability pores and enhancing the release of various pro-apoptotic proteins such as cytochrome-c [97]. Among the several regulators of the mitochondrial apoptotic pathway, the Bcl-2 family of proteins represents an important checkpoint in the intrinsic pathway of apoptosis [98]. It is comprised of several members including pro-apoptotic proteins like Bad, Bax, Bik, Bak and anti-apoptotic proteins such as Bcl-2, Bcl-X_L and Mcl-1 [99] [24, 98]. The founding member Bcl-2 was first identified as a proto-oncogene in low grade B cell lymphomas and follicular lymphomas [100, 101]. Soon it was determined that Bcl-2 did not enhance cell proliferation like other classical oncogenes, but instead blocked cell death [102]. The characteristic of this family is the

presence of at least one of four conserved motifs in each of its member, known as Bcl-2 homology (BH) domains (BH1 to BH4) [98]. The pro-apoptotic members of the Bcl-2 family such as Bad, Bim, Bid and Bik contain only the BH3 domain. The anti-apoptotic members, on the other hand, contain at least BH1 and BH2 domains. The anti-apoptotic members like Bcl-2 and Bcl-X_L possess all four BH domains. The interactions between pro and anti-apoptotic Bcl-2 family members regulate the release of cytochrome c but the exact mechanism is poorly understood [103-105] [98]. 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 [106-108].

Though a tight balance between the degree of mitosis and apoptosis is considered critical, an important aspect of tissue homeostasis is the clearance of dead cells from the body. A mismatch between execution of apoptosis and disposal of these cells may thus contribute to disease pathogenesis [109]. Thus programmed cell clearance is an equally important mechanism for removal of apoptotic cells from body [110].

Ca^{++} signaling

Ca^{++} signaling is crucial throughout the life of an organism and impacts numerous cellular processes ranging from fertilization, mitosis, cell proliferation, differentiation, gene transcription, muscle dynamics and cell death [111-114]. Underlying the great versatility and effectiveness of Ca^{++} signaling is the thousands of fold of gradient maintained by cells between its intracellular (nM) and extracellular (mM) concentrations (Reviewed in [115]). The great diversity in Ca^{++} signaling emerges from its ability to alter local electrostatic fields and an extensive repertoire of molecular components that it

interacts with and utilizes in the signaling. Since Ca^{++} itself can not be altered chemically, cells employ various ways to control it such as compartmentalization, chelation or extrusion affecting gradients across biological membranes. Some of the important organelles involved in Ca^{++} regulation are endoplasmic reticulum (ER), sarcoplasmic reticulum (SR), endosomes, Golgi vesicles, lysosomes and mitochondria [116]. Increases in cytoplasmic Ca^{++} levels are achieved using various mechanisms including Ca^{++} release from the internal stores (such as ER and SR) using various channels such as inositol 1,4,5-triphosphate receptors and ryanodine receptors and voltage or ligand operated plasma membrane Ca^{++} channels. Likewise, Ca^{++} is extruded from the cytoplasm by plasma membrane Ca^{++} ATPase pumps, Na^{+}/ Ca^{++} exchangers and SR/ER Ca^{++} ATPase pumps (SERCA).

The spatial and temporal aspects of Ca^{++} waves add further diversity to the downstream signaling [117, 118]. Thus to get an idea about the functional significance of Ca^{++} waves, a local pulse of Ca^{++} created at synaptic nerve endings is enough to stimulate the release of neurotransmitter vesicles or trigger muscle contraction. Likewise intercellular waves of Ca^{++} created within the ciliary epithelium in the lungs enable them to beat at a desired frequency in order to expel mucus from the airways [119]. Therefore the Ca^{++} signaling network is modulated by various means such as speed, amplitude, timing and spatio-temporal patterning.

Another important means adopted by cells to regulate Ca^{++} signals is through a variety of proteins that have been tailored to interact with Ca^{++} over a wide range of affinities. The purpose of these interactions could simply be to buffer the excess Ca^{++} or to act as a sensor to trigger various downstream signaling events. Thus proteins such as

parvalbumin and calbindin do not undergo much conformational change on binding Ca^{++} and act as buffers or Ca^{++} transporters. Another group of proteins, which serves as Ca^{++} sensors, undergo significant changes in protein shape and function with Ca^{++} binding and are particularly important in signal transduction [120]. This family of proteins is characterized by a structural Ca^{++} binding motif called an EF hand motif which is named so because a three dimensional arrangement of these motifs resembles the thumb, index and middle fingers of a hand [121]. Calmodulin, Troponin C and S100 family of proteins are important adapter proteins in this family [122]. Ca^{++} binding to EF hand motifs causes pronounced conformational changes in these proteins, allowing them to modulate numerous downstream targets. Thus troponin C regulates muscle contraction and CaM regulates several processes like muscle contraction, metabolism, gene transcription, ion channel functioning and cell death or survival.

The role of Ca^{++} in apoptosis is complex. The duration and extent of an intracellular Ca^{++} change may determine whether a cell survives or dies [123]. Sustained elevation of intracellular Ca^{++} or potentiation or inhibition of Ca^{++} currents can result in cell death [124-126]. On the other hand Ca^{++} ions have also been reported to exert protection against cell death [127-130]. How Ca^{++} triggers specific signals to effect cell survival or cell death is still not clearly understood. High intracellular Ca^{++} can activate hydrolytic enzymes such as proteases, nucleases and lipases [131, 132]. Endonuclease-mediated DNA degradation has long been reported to be dependent on Ca^{++} [133]. Ca^{++} is also believed to trigger mitochondrial dysfunction that triggers a cascade of caspase activation leading to apoptotic cell death.

Mitochondria are thought to represent an important regulator of Ca^{++} within the cells. Like ER it can store large amounts of Ca^{++} (mM) and thus reduce the cytosolic Ca^{++} by sequestering the ion. It however utilizes mechanisms for Ca^{++} regulation distinct from those in the ER. While Ca^{++} readily diffuses through the outer mitochondrial membrane, it crosses the inner mitochondrial membrane via mitochondrial Ca^{++} channels (MiCa) which bind Ca^{++} with very high affinity, imparting high Ca^{++} selectivity in spite of a low cytoplasmic Ca^{++} concentration [134]. Mitochondria can utilize Na^{+}/ Ca^{++} antiporter as well to sequester Ca^{++} in cases of Na^{+} overload. Likewise for extrusion of Ca^{++}, mitochondria utilize various mechanisms like an energy dependent pump linked to H^{+}/Na^{+} exchange or energy independent Ca^{++} release through permeability transition pores (PTP) [135]. PTP can be induced by oxidative stress or intracellular Ca^{++} overload causing membrane depolarization, mitochondrial swelling and rupture of the outer mitochondrial membrane [105, 136]. This is followed by the release of pro-apoptotic proteins from mitochondrial intermembrane space, such as cytochrome c [137, 138], Smac/DIABLO [139, 140], caspases [141, 142], endonuclease G [143] and apoptosis inducing factor [144]. In this regard, the Bcl-2 family members seem to play an important regulatory role. The anti- and pro-apoptotic members of this family may form homodimers or heterodimers to modulate the formation of mitochondrial permeability pores and release of apoptosis-inducing proteins such as cytochrome c [145-147]. Further, persistent depolarization of the mitochondrial membrane also inhibits respiration and depletes ATP which may kill cells by apoptosis or necrosis [105, 148].

Another important mediator in Ca^{++} homeostasis and Ca^{++} induced cell death are intracellular stores of Ca^{++} in the ER. Because of the close proximity of IP3-gated Ca^{++}

channels of the ER to the mitochondria, the ER bound Ca^{++} is also considered as a possible inducer of apoptosis [149]. Mobilization of Ca^{++} from these intracellular stores to mitochondria opens up its permeability transition pores leading to release of pro-apoptotic factors like cytochrome-c [149]. The role of increased intracellular free Ca^{++} in apoptosis was supported by using an inhibitor of sarcoendoplasmic reticulum Ca^{++}-ATPases, thapsigargin, which depleted ER Ca^{++} and induced DNA fragmentation and cell death [150, 151]. Bcl-2, which localizes to mitochondria, nuclear envelope as well as ER [152], appears to exert its anti-apoptotic effects, at least in part, by maintaining ER Ca^{++} homeostasis. Thus Bcl-2 over expression was shown to decrease thapsigargin (TG) mediated increases in intracellular Ca^{++} by reducing ER Ca^{++} efflux thus blocking the induction of apoptosis [153, 154]. Calreticulin, another Ca^{++} binding protein located in the ER, further supports the role of ER Ca^{++} in apoptosis [155]. Over expression of calreticulin resulted in increased storage of Ca^{++} within the ER and enhanced susceptibility to apoptosis [156, 157]. On the other hand calreticulin-deficient cells inhibited IP3 dependent Ca^{++} release and were found to be resistant to apoptosis [157, 158].

Thus the importance of Ca^{++} in apoptosis has been well established over the years whereby modulation of effectors of Ca^{++} such as calreticulin, calcineurin and CaM have been shown to regulate cell death signaling.

Calmodulin

Calmodulin (CaM) is a 17 kDa, dumbbell shaped, Ca^{++} binding protein that is ubiquitously expressed in the cells and has changed only slightly through evolution [159,

160]. It consists of four EF hand motifs, two at each end, separated by a helical linker region. The C-terminal EF hand motifs have three to five fold higher affinity for Ca^{++} than the N-terminal ones [161]. Further, in the absence of Ca^{++}, the N-terminal domain adopts a closed confirmation whereas the C-terminal domain is in a semi open confirmation with partial exposure of a hydrophobic patch. Thus the C-terminal domain of CaM could be accessible for target proteins even in the absence of Ca^{++} [162]. Thus CaM interacts with its target proteins in both Ca^{++} dependent as well as independent manners. Binding of Ca^{++} to the EF hand motifs induces a conformational change in CaM, exposing hydrophobic surfaces, which then interact with a variety of proteins triggering events such as release of auto inhibitory domains, remodeling active sites and protein dimerization [163, 164]. Likewise affinity of Ca^{++} binding to CaM is increased by about tenfold after CaM-target protein interaction [165]. Hundreds of proteins containing CaM recruitment sites have been described and are characterized by basic and bulky hydrophobic residues surrounded by aromatic amino acids [166].

Numerous studies have identified the importance of temporal and spatial associations between changes in Ca^{++} and the function of CaM. Changes in intracellular Ca^{++} have been shown to affect the cellular location of CaM including redistribution from the cytosol to the nucleus in a stimulus dependent manner in neurons, smooth muscles and pancreatic acinar cells [167-169]. Likewise, an increase in intracellular Ca^{++} and CaM function was identified in fibroblasts during wound healing, indicating a direct correlation between Ca^{++} surge and CaM signaling [170]. CaM not only acts as a transducer of Ca^{++} signals but also modulates intracellular Ca^{++} concentration. It does so

by regulating the plasma membrane Ca^{++} ATPase, various ion channels, ryanodine receptors, IP3 receptors and cyclic nucleotide gated Ca^{++} channels[171-173].

Several studies have demonstrated the importance of CaM in apoptosis. Expression of a CaM gene was found to be increased during glucocorticoid and Fas-mediated apoptosis [174, 175]. Inhibition of CaM has been shown to protect neurons against toxicity of excitatory amino acids [176]. Further various downstream substrates of Ca^{++}/CaM have been studied extensively and found to be important in the modulation of apoptotic pathways. Calcineurin, a Ca^{++}/CaM dependent serine/threonine phosphatase, has been reported to mediate neuronal apoptosis by dephosphorylating Bad, a pro-apoptotic protein or by activating nitric oxide synthase that exerts toxic effects [125, 177]. Death associated protein kinase (DAP-kinase) is a Ca^{++}/CaM dependent serine/threonine kinase which has been shown to be a positive regulator of apoptosis in response to various stimuli such as Fas and TNF-ά [178, 179]. Calmodulin dependent protein kinase II (CaMKII) is another downstream target of CaM which has been implicated in both pro- as well as anti-apoptotic functions. Thus inhibition of CaMKII by KN93 induces apoptosis and/or enhances the sensitivity to apoptosis of numerous cancer cell lines [180-182]. In contrast, recently CaMKII was reported to be essential for apoptotic cell death induced by toxins like microcystin [183]. CaM antagonists have been shown to induce apoptosis in cancer cell lines [184-187]. Interestingly, CaM has recently been shown to interact directly with the Fas receptor and CaM-antagonists were found to induce apoptosis in cholangiocarcinoma cells in a Fas-related manner [56, 187]. However, the effects of CaM antagonists differ among the cell types. In CD4+ T cells from AIDS patients, CaM antagonists have been reported to protect against apoptosis

[188]. The CaM antagonist, TFP, on the other hand, has been reported to induce apoptosis in some cell lines [184-186, 188]. Thus the signaling events triggered by Ca^{++}/CaM and downstream targets that are critical in apoptotic signaling are still unclear and the studies presented here are focused on delineating the role of CaM in apoptosis.

Cholangiocarcinoma

Cholangiocarcinoma is a fatal tumor arising from the epithelial lining of intra-hepatic or extra-hepatic bile ducts. The incidence of cholangiocarcinoma in the USA has increased persistently over the last two decades but the prognosis has not improved [189, 190]. The etiology of cholangiocarcinoma remains unclear, although chronic inflammation as observed in primary sclerosing cholangitis or chronic hepatobiliary parasitic infections, are associated with increased risk of cholangiocarcinoma [191-193]. The 5 year relative survival rate of the patients diagnosed with cholangiocarcinoma is less than 9%, with total surgical resection being the only curative treatment option available so far (*). However, the vast majority of patients do not qualify for this option because by the time they seek medical help, the disease has progressed to an advanced stage which is not amenable to surgical cure. Conventional chemotherapy and radiotherapy are ineffective in prolonging the survival in these patients [194, 195]. Thus there is real need to identify novel molecular targets that would impact clinical outcomes favorably. Resistance to apoptosis has been implicated as one of the important molecular mechanisms in the pathogenesis of cholangiocarcinoma [196, 197]. Apoptosis regulatory proteins, such as Fas, FasL, c-FLIP, Bcl-2, cyclooxygenase-2 (COX-2), protein kinase

B/AKT and Ca^{++}/ Calmodulin have been studied extensively as potential preventive and therapeutic targets in cholangiocarcinoma.

Down-regulation of one of the death receptors, Fas (CD-95), has been reported to correlate with histological de-differentiation, vascular invasion, tumor size, and short patient survival [198, 199]. Thus as the disease progresses and the pathology changes from well to poorly differentiated, the expression of Fas goes down and the disease acquires an apoptotic resistant phenotype. Accordingly, increased expression of Fas by interferon-γ (IFN-γ) has been shown to render low Fas expressing human cholangiocarcinoma cells more sensitive to apoptosis [187]. IFN-γ was found to upregulate many apoptosis-related molecules, including Fas, caspase-3, caspase-4, caspase-7, caspase-8 and Bak, thus facilitating Fas-mediated caspase cleavage, cytochrome-c release and Bax translocation. Further, tamoxifen (TMX) and trifluoperazine (TFP), both potent calmodulin antagonists, can induce apoptosis in cholangiocarcinoma cells in culture and decrease tumor cell growth in vitro in a Fas related manner and IFN-γ further enhanced the sensitivity of the cells to these agents [55, 200]. Thus there seems to be a cross talk between CaM and Fas pathway and both represent attractive therapeutic targets.

Transcription of the *Fas* gene has been shown to be affected by mutant forms of p53 [201]. Thus in the presence of mutant *p53,* Fas messenger RNA and surface protein expression was found to be down-regulated which correlates with partial protection against Fas-dependent cell death [201]. Further bile duct carcinomas but not the normal and dysplastic biliary tract epithelium showed nuclear expression of p53 [199, 202].

These studies imply that p53 expression is a late event in the development of cholangiocarcinoma.

The expression of FasL has also been demonstrated to be up-regulated in the early stages of cholangiocarcinoma, but found to be decreased in moderate to poorly differentiated tumors [198]. Thus, it has been proposed that the increased expression of FasL on cholangiocarcinoma cells provides a mechanism for immune evasion by inducing apoptosis of nearby lymphocytes [95, 203]. Therefore down-regulation of Fas and up-regulation of FasL in cholangiocarcinoma cells may reflect a mechanism to evade immune surveillance [198, 199]. Thus Fas expression seems to be important in the progression of cholangiocarcinoma and Fas expression may serve as a molecular biomarker and potential target for cholangiocarcinoma therapy.

c-FLIP is an anti-apoptotic protein that interferes with death receptor mediated apoptosis signaling by interfering with recruitment and/or activation of initiator caspases in the DISC. Expression of c-FLIP has been shown to be elevated in cholangiocarcinoma cells and its down regulation sensitizes cholangiocarcinoma cells to apoptosis [187, 203, 204]. Thus like in many other cancers, FLIP may be responsible for decreased apoptotic sensitivity of cholangiocarcinoma cells and could be used as a potential therapeutic target. Another important anti-apoptotic protein, Bcl-2 has also been studied in cholangiocarcinoma and found to be increased in malignant cholangiocytes [205]. Bcl-2 expression was reported to be inversely related to vascular invasion, lymph node metastasis, perineural invasion, aberrant p53 expression and apoptotic cell death in cholangiocarcinoma samples from patients [206]. Furthermore, well or moderately

differentiated carcinomas were found to express Bcl-2 more frequently than poorly differentiated carcinomas [206].

Recently, another Bcl-2 family protein, myeloid cell leukemia protein 1 (Mcl-1), which regulates the mitochondrial apoptosis pathway, has been found to correlate with progressive disease phenotype [207, 208]. The proinflammatory cytokine IL-6 [209] and bile acids have been reported to increase Mcl-1 [210]. The expression of Mcl-1 was found to be elevated in biliary dysplasia, invasive cholangiocarcinoma, and three cultured human cholangiocarcinoma cell lines [207] and was reported to mediate the resistance of these cells to TRAIL-induced apoptosis [208]. Consistently, down-regulation of Mcl-1 by small-interfering-RNA (siRNA) sensitized the cells to TRAIL-mediated apoptosis [208]. In addition, flavopiridol, an anticancer drug that rapidly down-regulates Mcl-1, also sensitized cells to TRAIL cytotoxicity.

Cyclooxygenase is the key enzyme in the conversion of arachidonic acid to prostaglandins [211]. COX-1 is expressed normally in most tissues whereas COX-2, an inducible isoform, is not expressed in most tissues under normal physiologic conditions. Recent studies have suggested that COX-2 may play a critical role in regulating cholangiocarcinoma tumor cell apoptosis [212-214]. Induction of COX-2 has been demonstrated in cholangiocarcinoma cells [215-217], that leads to prostaglandin E_2 overproduction which in turn inhibits Fas-mediated apoptosis in human cholangiocarcinoma cells. Prostaglandin E2 was also found to up-regulate the expression of Mcl-1 [212]. Consistent with this, a COX-2 inhibitor, NS-398, restored the sensitivity of cholangiocarcinoma cells to Fas-mediated apoptosis, apparently by down regulating Mcl-1 [212]. COX-2 inhibition resulted in inhibition of the growth of cultured rat

cholangiocarcinoma cells in a dose-dependent manner and suppression of anchorage-independent growth of the cells in soft agar [216].

AKT, also called protein kinase B, is an important survival kinase that has been shown to play an important role in cholangiocarcinoma pathogenesis [197, 204]. Several studies have suggested that increased phosphorylation of AKT is associated with increased FLIP expression and decreased sensitivity of cells to apoptosis [218-220]. Further, cholangiocarcinoma tissue samples from patients were reported to have increased phosphorylation of AKT compared to adjacent non-neoplastic tissues [204]. Activation of AKT has also been shown to regulate Fas sensitivity in many cancer cells [221, 222] and its inhibition sensitizes the cells to Fas-mediated apoptosis [223, 224]. The ability of AKT inhibitors to enhance Fas-mediated apoptosis appears to involve inhibition of FLIP [204, 225]. Inhibition of AKT has also been observed with the COX-2 inhibitor, celecoxib, which enhances apoptotic sensitivity of cholangiocarcinoma cells. These observations further support a multicomponent signaling crosstalk system between AKT, COX-2, Mcl-1 and Fas signaling pathways in regulating cholangiocarcinoma tumorigenesis.

Lack of early diagnosis and effective therapies make cholangiocarcinoma a highly lethal cancer with high morbidity and mortality. Better understanding of the pathophysiology of cholangiocarcinoma is essential for the development of novel strategies for prevention, early diagnosis and therapy. The Fas pathway, one of the extensively studied and well characterized apoptotic pathways, may provide a novel avenue for early diagnosis and possibly effective therapy for cholangiocarcinoma. Our group along with several others have identifed a correlation between expression of the

cell surface Fas receptors and disease progression in cholangiocarcinoma patients such that low Fas expression is correlated with progressive disease, suggesting that it could be exploited potentially as a biomarker [56, 198, 199]. Induction of apoptosis by targeting the Fas pathway inhibits cholangiocarcinoma cell growth and tumorigenesis, indicating its potential as a target for therapy as well. Further, CaM-antagonists, such as TMX and TFP, have also been shown to induce apoptosis in cholangiocarcinoma cells in a Fas-dependent manner and inhibit cholangiocarcinoma tumorigenesis in nude mice xenografts, making them probable agents for chemotherapy [55, 56, 187, 188, 203]. Although a variety of apoptosis modulators such as Fas, Bcl-2, Mcl-1, COX, AKT and CaM are implicated in cholangiocarcinoma pathogenesis, we focused on the biology of Fas pathway with emphasis on newly identified role of CaM in this signaling for several reasons. Firstly, both Fas and CaM pathways have been studied individually to great depths and have been established as potential targets in cholangiocarcinoma model by our group as well as others. Secondly, CaM-antagonists such as, TMX, are well tested drugs with a favourable safety profile and thus can be translated easily for use in patients. Lastly, understanding the biology of CaM in Fas signaling pathway will not only enhance our understanding of the relatively poorly characterized pro-survival arm of the Fas pathway but will also provide us with potentially new therapeutic targets that could be used either alone or in combination with CaM-antagonists such as TMX in cholangiocarcinoma.

The studies presented in this dissertation are aimed at characterizing the role of CaM in Fas pathway and the molecular mechanisms involved in CaM-antagonist-induced apoptosis and inhibition of cholangiocarcinoma tumorigeneis in nude mice. As a

part of this characterization, we identifed a direct, Ca^{++} dependent interaction between CaM and an anti-apoptotic protein, c-$FLIP_L$, but not with other DISC associated protein, such as FADD, caspase-8 and $FLIP_S$. The overall hypothesis of this project is that CaM interacts directly with c-FLIP to modulate Fas-induced signaling in cholangiocarcinoma cells and in turn regulate tumorigenesis.

The characerization of the CaM-FLIP binding has been discussed in the manuscript entitled " Calmodulin binding to cellular FLICE like inhibitory protein modulates Fas-induced signaling". The molecular mechanisms of CaM-antagonist, TMX-induced apoptosis and the importance of CaM-FLIP binding in TMX mediated apoptosis has been discussed in the manuscript entitled " Molecular mechanisms of Tamxifen therapy for cholangiocarcinoma: Role of Calmodulin".

CALMODULIN BINDING TO CELLULAR FLICE LIKE INHIBITORY PROTEIN MODULATES FAS-INDUCED SIGNALING

by

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Biochemical Journal

Format adapted for dissertation

ABSTRACT

We and others have demonstrated that Fas-mediated apoptosis is a potential therapeutic target for cholangiocarcinoma. Previously, we reported that calmodulin (CaM)-antagonists induced apoptosis in cholangiocarcinoma cells through Fas-related mechanisms. Further, we identified a direct interaction between CaM and Fas with recruitment of CaM into the Fas-mediated death inducing signaling complex (DISC), suggesting a novel role for CaM in Fas signaling. Therefore, we characterized the interaction of CaM with proteins recruited into the Fas-mediated DISC, including Fas associated death domain containing protein (FADD), caspase-8 and cellular FLICE like inhibitory protein (c-FLIP). A Ca^{++}-dependent direct interaction between CaM and $FLIP_L$, but not FADD or caspase-8, was demonstrated. Further, a 37.3±5.7% increase (n=6, p=0.001) in CaM-FLIP binding was observed at 30 minutes after Fas stimulation which returned to baseline after 60 minutes and correlated with Fas-induced increase of intracellular Ca^{++} that peaked at 30 minutes and decreased gradually over 60 minutes in cholangiocarcinoma cells. CaM-antagonist, trifluoperazine (TFP), inhibited Fas-induced increase in CaM-FLIP binding concurrent with inhibition of ERK phosphorylation, a downstream signal of FLIP. Direct binding between CaM and $FLIP_L$ was demonstrated using recombinant proteins and a CaM binding region was identified in the amino acids 197-213 of $FLIP_L$. Compared to over expression of wild type $FLIP_L$ that resulted in decreased spontaneous as well as Fas-induced apoptosis, mutant $FLIP_L$ with deletion of

CaM binding region resulted in increased spontaneous and Fas-induced apoptosis in cholangiocarcinoma cells. Understanding the biology of CaM-FLIP binding may provide new therapeutic targets for cholangiocarcinoma and possibly other cancers.

Key words: Fas, Cholangiocarcinoma, Calmodulin (CaM), Death inducing signaling complex (DISC), FLICE like inhibitory protein (FLIP), Trifluoperazine (TFP).

INTRODUCTION

Apoptosis or programmed cell death is a tightly regulated process by which abnormal cells are removed from the body without generating an inflammatory response. The hallmark of this process is the activation of specific proteases called caspases, to effect DNA fragmentation and ultimately cell death [1]. This process is crucial not only during embryonic development for proper organogenesis but also throughout life to maintain cellular homeostasis. Too much or too little apoptosis disturbs this homeostasis, leading to various pathological conditions such as autoimmunity, neurodegeneration and cancer [2]. Induction of apoptosis has been used as a therapeutic modality in many cancers [3-6]. Previous studies from our group and others have demonstrated the importance of Fas-mediated apoptosis in tumorigenesis in cholangiocarcinoma, which is a fatal tumor arising from intra-hepatic or extra-hepatic biliary epithelium [7-11].

Fas (APO-1/CD-95) is a well characterized member of the tumor necrosis factor (TNF) superfamily of death receptors [12]. In response to the cognate ligand, FasL, it undergoes oligomerization and recruits the adaptor proteins FADD, which in turn recruits caspase-8 and/or c-FLIP, to form the death inducing signaling complex (DISC). Whether caspase-8 or its enzymatically inactive homologue, c-FLIP, is recruited into the DISC

determines whether death or survival signals are transmitted through the DISC [13-16]. c-FLIP has been described largely as an anti-apoptotic protein that interrupts apoptotic signaling by interfering with recruitment and/or activation of an initiator caspase, like caspase-8, into the DISC [17]. At the mRNA level, 11 distinct splice variants of c-FLIP have been reported [18]. At the protein level, however, two isoforms; a long isoform, $FLIP_L$ (55kDa), and a short isoform, $FLIP_S$ (26kDa), have been identified and extensively studied. $FLIP_L$ shares structural homology with caspase-8 and contains two death effector domains (DEDs) at its N-terminus and a long C-terminal tail. However, it lacks cystein residues in the catalytic subunit that are essential for caspase activity [15, 16]. Because of the structural similarity between these two proteins, it has been suggested that FLIP may have dual functions in apoptotic signaling [19]. On one hand, it interferes with the recruitment and/or activation of caspase-8 in the DISC [17, 20]. On the other hand, it may also facilitate activation of caspase-8 by forming a heterodimer with it, which helps achieve the initial cleavage step of procaspase-8 [19, 21]. The short isoform, $FLIP_S$, is believed to be exclusively anti-apoptotic. In situations where the expression of caspase-8 is stable, the expression levels of $FLIP_L$ are believed to be important in determining its role in apoptosis signaling [22, 23]. However, the precise mechanism that enables $FLIP_L$ to switch these signals and modulate the sensitivity of cells to Fas-induced signaling is not clearly understood.

We have previously demonstrated that calmodulin (CaM) antagonists induce apoptosis through a Fas-related mechanism in a cholangiocarcinoma tumor model [7, 10]. CaM is a 17 kDa, dumbbell shaped, primarily cytoplasmic protein that binds calcium (Ca^{++}) through EF hand motifs at each of its globular ends [24, 25]. Ca^{++} binding to CaM

exposes a hydrophobic linker region between two globular domains of CaM that binds with a variety of target proteins and mediate their effects. Several studies have demonstrated the importance of CaM in apoptosis and the expression of a CaM gene was found to be increased during glucocorticoid and Fas-mediated apoptosis [26, 27]. CaM antagonists have been shown to induce apoptosis in several cancer cell lines [10, 28, 29]. However, the effects of CaM antagonists differ among cell types. In CD4+ T cells from AIDS patients, the CaM antagonists trifluoperazine (TFP) and tamoxifen (TMX) have been reported to protect against apoptosis [30]. TFP, on the other hand, has been reported to induce apoptosis in some cell lines [7, 10, 29].

Recently, studies from our group demonstrated a direct binding between CaM and the Fas receptor that was found to be regulated during Fas-induced apoptosis [31]. The CaM-Fas interaction is unique in that the other members of the death receptor family such as TNFR-1 and TNF-related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1/DR4, TRAIL-R2/DR5 were not found to interact with CaM. Furthermore, CaM was found to interact with Fas death domain (DD) through a classic Ca^{++} dependent CaM binding motif termed 1-5-10 motif. CaM interacts with target proteins in both Ca^{++} dependent as well as independent manners [32]. Specific characteristics such as a net positive charge, moderate to high helical hydrophobic moment and moderate hydrophilicity, have been identified to predict the CaM binding region in a given sequence [33]. Based on the structural analyses of various CaM binding proteins, three classes of CaM-binding motifs have been described. These include two motifs for Ca^{++} dependent binding, termed 1-8-14 and 1-5-10, with numbers indicating the conserved

hydrophobic residues in a sequence and a consensus sequence for Ca^{++} independent binding, called an IQ motif [34].

The binding between CaM and Fas that is regulated during Fas-mediated apoptosis, prompted us to further study the interaction of CaM with other proteins in the Fas-induced DISC, since Fas-stimulated DISC formation is critical in Fas-mediated apoptosis. Here we report that CaM specifically binds with $FLIP_L$ but not other members of Fas-induced DISC including FADD and caspase-8. CaM binds specifically with the long isoform of c-FLIP, the interaction is Ca^{++} dependent and regulated in response to activation of Fas. Further, the CaM antagonist TFP inhibits CaM-FLIP binding with concurrent inhibition of ERK phosphorylation. In addition, CaM-FLIP binding is direct and the CaM binding domain localized to $FLIP_L$ amino acids 197-213. Overexpression of $FLIP_L$ lacking this CaM binding region increased both spontaneous and Fas-stimulated apoptosis in chaolangiocarcinoma cells as compared to wild type $FLIP_L$ over expressing cells, suggesting that CaM-FLIP binding is important in mediating survival signals by c-FLIP.

MATERIALS AND METHODS

Cells, antibodies and reagents

The human cholangiocarcinoma cell line Sk-ChA-1 was provided by Dr. A. Knuth (Ludwig Institute for Cancer Research, London, UK). Cholangiocarcinoma cells were grown in RPMI-1640 medium with 2mM L-glutamine, 5 units/ml penicillin; 5µg/ml streptomycin and 10% heat inactivated fetal bovine serum. For activation of Fas pathway, cells were seeded at 70-80% confluence, followed by treatment with Fas agonist antibody

(CH-11) over 60 minutes. Antibodies include: monoclonal anti-FLIP antibody, NF-6 (Alexis Corp., San Diego, CA), polyclonal anti-FLIP and anti-FADD antibodies (BD Biosciences, San Jose, CA), mouse monoclonal anti-caspase-8, rabbit polyclonal phospho-ERK and ERK (p44/42) antibodies (Cell Signaling, Boston, MA), Fas activating antibody, clone CH-11 (Upstate Biotechnology, Charlottesville, VA), Alexa Fluor secondary antibodies; goat anti-mouse IgG (Alexa Flour® 488) and goat anti-rabbit IgG (Alexa Flour® 594) (Molecular probes, Invitrogen, Carlsbad, CA), anti-GAPDH antibody (Research Diag. Inc, Concord, MA), rabbit polyclonal anti-CaM antibody (Zymed laboratories, San Francisco, CA) and goat polyclonal anti-GST-HRP antibody (Amersham Biosciences, Piscataway, NJ). The monoclonal antibody to CaM was developed as described [35].

Protein pull down

Protein pull down with Calmodulin-Sepharose® 4B (CaMS, Amersham Biosciences, Piscataway, NJ) or control Sepharose CL-4B beads (CS, Sigma Aldrich, St Louis, MO) was performed as described previously [31]. Briefly, cholangiocarcinoma cells were lysed in lysis buffer [20 mM Tris, 150 mM NaCl, 1% Triton-X100, 10% Glycerol, 1mM phenylmethylsulphonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA) free protease inhibitor cocktail from Roche, 1mM sodium fluoride (NaF), 1mM sodium orthovanadate] and 800µg of extracted proteins were incubated with 60 µl 1:1 slurry of beads overnight at 4ºC. The beads were washed 5 times with lysis buffer and proteins were eluted in 2X sodium dodecyl sulfate (SDS) buffer containing 10mM ethylene glycol tetraacetic acid (EGTA).

Co-immunoprecipitation

Seize™ primary mammalian immunoprecipitation kit (Pierce Biotechnology, Rockford, IL) was used for covalent conjugation of anti-CaM antibody to the activated agarose beads. Briefly, 50µg anti-CaM or mouse IgG1 control antibodies were used for conjugation with 100µl slurry of activated agarose beads in a column according to the manufacturer's protocol. Cell lysates were incubated overnight at 4ºC in these columns followed by washing and elution of the proteins from the columns as per the protocol. The beads were boiled in 2X SDS sample buffer.

Western blot analysis

Whole cell extracts (50µg proteins) or eluted proteins from pull down or immunoprecipitation assays were separated by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) as described previously [22]. The proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA). For CaM, the membranes were fixed in 0.2% glutaraldehyde in tris buffered saline for 30 minutes. The membranes were blocked in 3% non fat dry milk in tris buffered saline with 0.05% Tween-20 for 1 hour at room temperature. Appropriate primary antibodies were added on to the membranes and incubated overnight at 4ºC followed by three washes for 10 minutes each. Horseradish peroxidase (HRP) conjugated secondary antibodies in the same blocking buffer were incubated for 1hour at room temperature and washed three times. Signals were detected using immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA) detection kit. The band intensities were analyzed by

densitometry using Adobe Photoshop Elements software (Adobe Systems Incorporated, San Jose CA).

Apoptosis assay

Apoptosis was induced with Fas activating antibody, CH-11, as described previously [22]. Briefly, $1x10^6$ cells were seeded in a 6 well plate and exposed to 500ng/ml of CH-11 antibody over 24 hours. Apoptosis was determined by staining the cells with Annexin V and propidium iodide (PI) using Annexin V- fluorescein isothiocyanate apoptosis detection kit (BD biosciences, Palo Alto, CA) and analyzed by flow cytometry according to manufacturer's recommendation. The percentage of cells that were Annexin V positive and PI negative was determined as apoptotic cells.

Immunofluorescence staining

Cholangiocarcinoma cells ($1x10^6$) were seeded on 18x18 mm glass cover slips in a 6 well plate. Subsequently, cells were washed with ice cold phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde in PBS and permeabilized using 0.5% TritonX-100 in PBS. After blocking with 1% bovine serum albumin in PBS, cells were incubated with primary antibody (a mouse monoclonal anti-CaM and a rabbit polyclonal anti-FLIP antibody, 10ug/ml in blocking buffer) at room temperature for 1 hour and then secondary antibody (Alexa Flour® 488 anti-mouse IgG or Alexa Flour® 594 anti-rabbit IgG, 10ug/ml in blocking buffer) for 30 minutes. Cells incubated with secondary antibodies alone were used as a negative control. Microscopic images were taken at 100X magnification using an Olympus IX 70 inverted microscope.

Expression and purification of GST-fusion proteins in E-coli

Expression and purification of GST-FLIP fusion proteins was performed as described previously[31]. The human c-$FLIP_L$ cDNA was amplified from a human umbilical vein endothelial cells library by PCR, cloned into pcDNA3 (Invitrogen Corp., Carlsbad, CA) and confirmed by sequencing. To express recombinant FLIP proteins *in vitro*, $FLIP_L$ cDNA was cloned into a GST vector (pGEX-5X-3, Amersham Biosciences, Piscataway, NJ) using blunt end ligation with restriction enzyme SmaI. The mouse $FLIP_L$ construct was a kind gift from Dr. Khosravi-Far R (Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA). The deletion mutants with human $FLIP_L$ were generated using restriction enzyme (EcoRI, ApoI, ClaI, AflII, HindIII, BamHI) - mediated digestion of cDNA followed by cloning into a GST vector. Delta FLIP deletion mutant with deletion of amino acids 197-213 was generated using restriction enzymes BamHI and AflII mediated digestion of $FLIP_L$ cDNA. All the mutants were confirmed by sequencing before expression into the bacterial system. The fusion proteins were expressed in *E-coli,* strain DH-5α, by inducing with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30ºC. The bacteria were lysed in GST lysis buffer (1X PBS, 50 mM EDTA, 10% glycerol, 0.5% aprotonin, 1mM Dithiothreitol **(**DTT), 1mM PMSF) with lysozyme and purified with a GST expression and purification kit (Amersham Biosciences, Piscataway, NJ).

Assay for intracellular Ca^{++}

Intracellular Ca^{++} was detected using FLUO-4-AM dye (Molecular Probes, Invitrogen, Carlsbad, CA) as described previously [36]. Briefly, $1x10^5$

cholangiocarcinoma cells were seeded in a 35mm dish with a 14 mm glass bottom well. Cells were washed with Hanks Balanced Salt Solution (HBSS) and incubated at room temperature with 5uM FLUO-4 AM dye dissolved in dimethyl sulfoxide (DMSO) followed by three washes of 5 minutes each with HBSS. After an initial image was recorded, Fas-activating antibody (CH-11, 500ng/ml) was added to the sample followed by imaging over 60 minutes with one image recorded per minute. All images were taken at 100X magnification using Olympus IX 70 inverted microscope with epifluorescence optics. IPLab Spectrum and Ratio from Scanalytics in Fairfax, VA were used for image acquisition and data analysis.

Generation of cholangiocarcinoma cells stably overexpressing WT or mutant (delta) $FLIP_L$

Stable over expression of $FLIP_L$ in cholangiocarcinoma cells was achieved using a lentiviral $FLIP_L$ expression vector (pLenti-FLIP) kindly provided by Dr. Shi-Yong Sun (Winship Cancer Institute, Emory University, Atlanta, GA, USA) [37]. The lentiviral vector expressing a $FLIP_L$ deletion mutant lacking CaM binding region (delta FLIP) was generated by using restriction enzymes BamHI and AflII to delete amino acids 197-213 from full length $FLIP_L$ cDNA. Infectious viral vector particles were generated by each expression vector into 293T cells together with the pΔ8.9 packaging construct and the Env pseudotyping contruct pmD.G, as described previously [38]. Cholangiocarcinoma cells were transduced with each of the vectors for exogenous expression of the WT FLIP and delta FLIP proteins, respectively.

Statistical analysis

The differences between two groups were analyzed by student t-test. For multiple groups, one-way analysis of variance and Student-Newman-Keuls tests were used to identify differences. Significance was defined as $p<0.05$.

RESULTS

CaM interacts with $FLIP_L$ but not with FADD and caspase-8

We have recently characterized CaM binding with Fas, a member of the TNF super family of death receptors, and this binding is regulated during Fas-induced apoptosis [31]. The interaction of CaM and Fas is unique, because CaM does not bind to other members of TNF family such as DR4, DR5 or TNF-R1. This exclusive interaction between CaM and Fas suggests a novel role for CaM in Fas signaling pathway which could be a mechanism to explain the variable effects of CaM-antagonists in inducing apoptosis in various cell lines. To further delineate the role of CaM in Fas-induced signaling, we determined if CaM interacted with other members of Fas-induced DISC. Protein pull down assays were performed with proteins extracted from cholangiocarcinoma cells using Calmodulin-Sepharose (CaMS) beads. Western blot analyses of proteins pulled-down by CaMS demonstrated the specific interaction of CaM with c-$FLIP_L$, but not with the other DISC associated proteins, FADD and caspase-8 (Fig.1A). In addition, CaMS beads did not pull down the short isoform of c-FLIP, $FLIP_S$. The binding between CaM and $FLIP_L$ was further confirmed in cholangiocarcinoma cells by co-immunoprecipitation (Co-IP) analysis with anti- CaM antibody (Fig. 1B). Similarly, co-immunoprecipitation of CaM and $FLIP_L$ was observed in Jurkat cells (data

not shown), suggesting that CaM/FLIP binding could be a universal phenomenon. The CaM/FLIP binding was further visualized by immunofluorescence staining. As depicted in Fig. 1C, CaM (shown in green color) and FLIP (shown in red color) co-localized (seen as a yellow color in merged image) in cholangiocarcinoma cells.

Interaction between CaM and $FLIP_L$ is Ca^{++} dependent

Since CaM interacts with its target proteins in both Ca^{++} dependent and independent manners, the interaction between CaM and FLIP was further characterized to determine whether the binding was affected by Ca^{++}. The effect of a Ca^{++} chelator, EGTA, on CaM-FLIP binding was determined. EGTA (2mM) inhibited the binding between CaM and $FLIP_L$, as decreased amount of $FLIP_L$ was pulled down by CaMS beads in protein pull down assays (Fig. 2A). $FLIP_s$ was not pulled down by CaMS, both in the presence and absence of EGTA. Similarly, EGTA decreased the amount of $FLIP_L$ co-precipitated with CaM in immunoprecipitation using anti-CaM antibody, further confirming the Ca^{++} dependence of CaM- $FLIP_L$ interaction *in vivo* (Fig. 2B).

CaM-FLIP binding is regulated in response to Fas activation

The biological significance of CaM-$FLIP_L$ binding in Fas-mediated apoptosis was further characterized in cholangiocarcinoma cells. We determined that treatment with Fas activating antibody (clone CH-11) over 24 hours induced significant apoptosis ($59\pm5\%$, $n=8$, $p<0.05$) in cholangiocarcinoma cells. We further determined whether Fas activation affected the CaM-FLIP interaction. Immunoprecipitation analysis of cholangiocarcinoma cells exposed to Fas activating antibody for 30 and 60 minutes

demonstrated that CaM-FLIP binding was increased significantly at 30 minutes and returned to basal level at 60 minutes (Fig, 3A). The band intensities for FLIP and CaM were measured using densitometry and the percentage change in the ratios of band intensities of FLIP with respect to the corresponding band intensities of CaM were used to determine the change in CaM and FLIP binding. As compared to untreated cells, a 37.3 ± 5.7% increase (n=6, p=0.001) in CaM-FLIP binding was identified in cells exposed to Fas activating antibody for 30 minutes and the binding was decreased to basal level at 60 minutes (Fig. 3B, right panel). Immunofluorescence staining for CaM (Green) and FLIP (Red) after stimulation of cholangiocarcinoma cells with Fas activating antibody for 30 minutes showed that CaM and FLIP co-localized in the same apparent discrete sub-cellular location, as opposed to the diffused cytoplasmic staining seen in unstimulated cells, further confirming that the CaM-FLIP binding is regulated in response to Fas activation (Fig 3C). Fas and c-FLIP are known to move towards the membrane compartment of the cell to form the DISC during Fas-induced apoptosis. Thus it is likely that CaM and FLIP are moving towards the membrane compartment of the cell in response to Fas activation. These findings further underscore the involvement of CaM in Fas-induced apoptosis.

Intracellular Ca^{++} increases in response to Fas activation

Intracellular Ca^{++} has been reported to increase in response to the induction of apoptosis [39]. We investigated the response of Ca^{++} in cholangiocarcinoma cells after Fas stimulation using FLUO-4 AM dye that stains intracellular Ca^{++}. The images were recorded and quantitated as described in the materials and methods section. An increase

in intracellular Ca^{++} that peaked at 30 minutes followed by a gradual decline over 60 minutes after Fas activation was observed in cholangiocarcinoma cells as shown in Fig.4. Thus the increased binding between CaM and FLIP at 30 minutes (Fig.3) following Fas stimulation paralleled the rise in intracellular Ca^{++} that peaked at 30 minutes in response to Fas-activating antibody, suggesting that the Ca^{++} dependence of CaM-FLIP binding is biologically significant.

Calmodulin-antagonist inhibits Fas-induced CaM-FLIP binding and ERK activity

TFP is a potent CaM-antagonist and we have shown previously that it induces apoptosis in cholangiocarcinoma cells in a Fas related mechanism [7, 10]. To further characterize the biologic relevance of CaM-FLIP binding in Fas mediated signaling, we determined the effect of TFP (10uM) on CaM-FLIP binding and the activity of extracellular-signal regulated kinase (ERK) pathway, a downstream signal that has been reported to mediate the survival pathway of FLIP in response to activation of death receptors [40]. Fas, extensively studied as a death receptor, is able to send survival signals as well through ERK activation and it is speculated that the final outcome of Fas stimulation depends upon which signaling pathway; pro-apoptotic or pro-survival, predominates in a given system [41, 42]. We found that pretreatment of cholangiocarcinoma cells with TFP (10 μM) for 30 minutes inhibited the Fas-stimulated increase in CaM-FLIP binding (Fig. 5A). The basal level of CaM-FLIP binding was also decreased by TFP. Further, we determined whether inhibition of CaM-FLIP binding affected the phosphorylation of ERK signaling (p44/42 MAPK). Exposure of cells to TFP for 30 and 60 minutes did not affect phosphorylation of ERK (pERK, Fig 5B,

extreme right panel). However, pretreatment with TFP caused decrease in phosphorylation of ERK after cells were exposed to Fas activating antibody (CH-11), although CH-11 alone did not affect phosphorylation of ERK at 30 and 60 minutes. Such inhibition of ERK phosphorylation by TFP paralleled its inhibition of CaM-FLIP binding. Thus, increased CaM-FLIP binding after Fas stimulation appears to be important in maintaining ERK phosphorylation.

Binding between CaM and $FLIP_L$ is direct

To determine whether CaM binds to $FLIP_L$ directly, purified recombinant $FLIP_L$ was used in protein pull down assay with CaM sepharose beads. Human $FLIP_L$ cDNA was cloned into a GST vector, expressed in *E. coli* and the proteins were purified using affinity purification. As shown in Fig 6A, GST-FLIP protein bound to CaMS but not to control sepharose (CS) beads, indicating a specific direct binding of CaM and FLIP. The binding between CaM and GST-FLIP was diminished in the presence of the Ca^{++} chelator, EGTA (Fig. 6B), confirming the Ca^{++} dependence of the binding between purified proteins. To exclude the possibility of nonspecific binding between CaM and GST, Factor Xa was used to cleave GST tag from the GST-FLIP fusion protein. Recombinant $FLIP_L$ protein cleaved from the GST-FLIP fusion protein bound specifically to CaMS beads (Fig. 6C). The direct binding between CaM and $FLIP_L$ was confirmed by using mouse $FLIP_L$ construct as well (data not shown).

Computer modeling of $FLIP_L$ structure and generation of GST-FLIP deletion mutants

Based on the structural characteristics of various CaM binding proteins, the putative CaM binding regions on a given sequence can be predicted using various criteria including the hydrophobic moment, net charge and propensity of the sequence to form alpha helical structure [33, 34]. To date, only the short isoform of c-FLIP ($FLIP_S$) has been crystallized [43] and hence the structure of the c-terminal tail of $FLIP_L$ is not known. Since $FLIP_L$ shares extensive sequence and probably structural homology with caspase-8, the structure of caspase-8 was used as a reference to determine the helical sequences in the three dimensional model of $FLIP_L$. Six alpha helices, shown as coiled ribbons, were identified in the c-terminal $FLIP_L$ sequence as potential CaM binding regions (Fig. 7A) and used to direct the generation of $FLIP_L$ deletion mutants using restriction enzymes mediated digestion of human FLIP cDNA as shown in Fig. 7B. The FLIP cDNA fragments obtained were cloned into a GST vector to obtain six N-terminal, one C-terminal and a delta FLIP deletion mutant with deletion of amino acids 197-213 from full length $FLIP_L$. After confirming the sequences of these GST-FLIP deletion mutants, proteins were expressed in *E-coli* using IPTG induction and confirmed by immunoblotting for GST and/or FLIP.

CaM binds to $FLIP_L$ between amino acids 197-213

The series of GST-FLIP deletion mutants (shown in Fig. 7B) were used in protein pull down assays to identify potential CaM binding motif on FLIP. We found that mutants N383, N280, N257, N233 N213 and C280 but not N196 bound with CaM (Fig. 8). Thus, the likely CaM binding site was narrowed to amino acids 197-213 on $FLIP_L$.

Further deletion of amino acids 197-213 (using restriction enzymes BamHI and AflII) from $FLIP_L$ cDNA showed reduced binding of this mutant with CaM (Fig. 8D), confirming that the region from amino acids 197-213 in $FLIP_L$ is important for binding with CaM. The short isoform, $FLIP_S$, contains 221 amino acids, with the N-terminal 202 amino acids identical to those of $FLIP_L$. Thus localization of CaM binding site on $FLIP_L$ to amino acids 197-213 explains the lack of CaM binding to $FLIP_S$. Further, the binding of fragment N213, the shortest $FLIP_L$ mutant to bind CaM was Ca^{++}-dependent (Fig 8B). Interestingly, although the CaM-$FLIP_L$ interaction was found to be Ca^{++} dependent, the classical CaM binding motifs, 1-8-14 and 1-5-10, observed in many proteins binding with CaM in Ca^{++} dependent manner [34] were not identified in this $FLIP_L$ sequence. This may categorize $FLIP_L$ as a novel interacting partner in a family of CaM binding proteins with a CaM binding motif not previously identified.

CaM-FLIP binding is important in mediating spontaneous as well as Fas-induced apoptosis by $FLIP_L$

$FLIP_L$ has been ascribed with anti- as well as pro-apoptotic functions in Fas signaling [19]. To determine the function of FLIP in cholangiocarcinoma cell lines and the importance of CaM- $FLIP_L$ binding in mediating $FLIP_L$ function, we stably over expressed wild type $FLIP_L$ (WT FLIP) and $FLIP_L$ lacking the CaM binding region (delta FLIP) in cholangiocarcinoma cells using the lentiviral expression vectors (Fig. 9A). Over expression of WT FLIP reduced both basal apoptosis and the sensitivity to Fas-induced apoptosis as compared to control (LacZ) cells (Fig. 9B). Even though the delta FLIP over expressing cells were found to be resistant to Fas-induced apoptosis as compared to control (LacZ) cells, their sensitivity to Fas-induced apoptosis was higher than WT FLIP

over expressing cells. Thus WT FLIP was found to be a more efficient inhibitor of Fas stimulated apoptosis than delta FLIP protein (Fig. 9B, mean±SE, n=8, $p<0.05$). Therefore CaM-FLIP binding seems to be important for the anti-apoptotic function of $FLIP_L$ in the Fas pathway. It is likely that the abrogation of CaM-FLIP binding may affect $FLIP_L$ such that there is inefficient recruitment and/or activity of $FLIP_L$ into the DISC. Interestingly, unlike WT FLIP that rendered cells more resistant to apoptosis under unstimulated conditions, basal apoptosis was increased significantly in cholangiocarcinoma cells that over expressed the delta FLIP protein, suggesting that CaM-FLIP binding is also an important determinant of spontaneous apoptosis under basal conditions.

DISCUSSION

CaM interacts with a variety of proteins such as calcineurin, CaM kinases, myosin light chain kinase, nitric oxide synthase and neuromodulin [34] and affects numerous signaling pathways such as inflammation, memory, muscle contraction, the immune response and ion channel functioning [44]. Recently, we reported a direct and dynamic interaction between CaM and Fas suggesting a novel role of CaM in Fas-mediated signaling. CaM binding to Fas is mediated through a classic Ca^{++} dependent CaM binding region termed, 1-5-10 motif, present in the cytoplasmic death domain of Fas [31]. The point mutation (V254N) in the cytoplasmic death domain of Fas in lpr^{cg} mice, whose phenotype is analogous to that of human autoimmune lymphoproliferative syndrome, results in the inability of Fas to form the DISC [45]. This same mutation also causes reduced binding between CaM and Fas as seen in osteoclasts from lpr^{cg} mice [46]. Considering these findings and the multitude of signaling pathways CaM is involved in, it

is likely that CaM plays an important but yet undetermined role in Fas mediated signaling. Consistently, we found that CaM is recruited into the Fas-mediated DISC [47]. Therefore, we studied the interaction of CaM with other proteins involved in Fas-induced DISC. Here we report that c-$FLIP_L$, a mediator in the Fas stimulated DISC, is a novel binding partner of CaM. We confirmed the direct binding between CaM and c-$FLIP_L$ by various methods including CaMS protein pull down, immunoprecipitation, epifluorescence and in vitro binding assays using recombinant proteins (Fig. 1, 2 and 6). The CaM-FLIP binding was increased in response to Fas activation and this increase in binding correlated with a rise in intracellular Ca^{++} in response to Fas activation, further corroborating a likely functional significance of this binding in cholangiocarcinoma cells (Fig 3A, 3B and 4). Interestingly, CaM does not interact with other homologues proteins of the DISC; FADD and caspase-8, which gives rise to the interesting possibility that CaM-Fas and CaM-FLIP binding may act as a counter mechanisms for Fas-FADD-caspase-8 assembly during DISC formation.

The CaM binding region was localized to amino acids 197-213 on $FLIP_L$ which also explains the lack of binding of CaM with c-$FLIP_S$ (short isoform of c-FLIP). $FLIP_S$ is a 26 kDa protein with 221 amino acids whose sequence differs from the long isoform, $FLIP_L$, from amino acid 203 to the C-terminus. Also this region lies outside the death effector domains (DEDs) of c-FLIP, further explaining the lack of CaM binding with other homologous proteins, FADD and caspase-8, which share DEDs as the homologous domain in the family. In addition, the CaM binding site on $FLIP_L$ does not appear to share the characteristics of any of the classical Ca^{++} dependent CaM binding motifs

described so far and thus $FLIP_L$ may be a novel target in a family of CaM binding peptides.

The precise role of CaM-FLIP interaction in regulating Fas-mediated apoptosis has yet to be fully elucidated. Here we showed that overexpression of WT FLIP decreased spontaneous as well as Fas-induced apoptosis in cholangiocarcinoma cells. However disruption of CaM-FLIP binding with (delta) FLIP rendered cholangiocarcinoma cells more sensitive to spontaneous and Fas-induced apoptosis as compared to WT FLIP over expressing cells. Thus, CaM-FLIP binding appears to be important not only for mediating anti-apoptotic effects of $FLIP_L$ in the Fas pathway, but also for maintaining survival signals under basal conditions. It is possible that the disruption of CaM-FLIP binding causes a conformational change in FLIP, which affects its recruitment into the DISC thus affecting its anti-apoptotic role in cholangiocarcinoma cells. Further investigations are warranted to define the mechanisms by which CaM-FLIP modulates cell survival and apoptosis under basal conditions and upon Fas stimulation.

We showed here that CaM-FLIP binding increased after 30 minutes of Fas activation in cholangiocarcinoma cells (Fig 3A and B). Further, CaM and FLIP were shown to co-localize in the same apparent discrete sub-cellular compartment after Fas activation. In combination, these observations demonstrated that the CaM-FLIP binding is regulated upon Fas stimulation, which might be important for mediating anti-apoptotic and/or pro-survival signaling by FLIP in response to Fas stimulation. Consistently, we showed here that the CaM-antagonist, TFP, interfered with Fas-induced increase in CaM-FLIP binding with concurrent decrease in phosphorylation of ERK, a pro-survival signal downstream of FLIP (Fig.5). It is possible that the functions of CaM are cell-type

specific, possibly paralleling the cell-specific apoptosis-inducing abilities of CaM-antagonists [10, 28-30].

Both CaM-Fas and CaM-FLIP interactions are Ca^{++} dependent. Therefore, there is a possibility that these interactions, by engaging CaM, affect the Ca^{++} flux in the vicinity of the membrane compartment of the cell. As opposed to Jurkat cells in which rise in intracellular Ca^{++} is comparatively a late phenomenon [39], cholangiocarcinoma cells showed a relatively rapid rise in intracellular Ca^{++} in response to Fas stimulation [Fig. 4]. Jurkat cells are known to be type II cells in which only minimal formation of the DISC is required to initiate apoptosis that proceeds mainly through the mitochondrial pathway [48]. Type I cells however are characterized by the formation of the DISC (to recruit caspase-8) which is required for the activation of downstream caspases. Over expression of $FLIP_L$ in cholangiocarcinoma cells interfered with caspase-8 activation and significantly reduced their sensitivity to Fas-mediated apoptosis, which is consistent with previous reports attributing an inhibitory role to c-FLIP in Fas-mediated apoptosis in cholangiocarcinoma cells [9]. Thus the cholangiocarcinoma cells that we used in the present studies appear to be type I cells in which modulation (with increased recruitment of c-FLIP) in the formation of the DISC abrogates apoptotic cell death. Therefore it is very likely that the CaM-FLIP interaction is important modulator of Fas-induced DISC which is the essential pathway for apoptotic cell death in cholangiocarcinoma cells.

Although Fas is well characterized as a death receptor in the apoptotic machinery, it can also activate non apoptotic signaling events [45]. Activation of Fas has been shown to induce cell proliferation and tissue regeneration as well [49] and c-FLIP is touted as one of the important determinants of downstream signaling initiated after Fas

activation. Fas-induced activation of ERK pathway has been shown to be independent of caspase activation such that caspase inhibition did not affect Fas-induced ERK activation [42]. This supports the notion that Fas-CaM-FLIP arm of the DISC might be important in modulating survival signals in response to Fas activation, independent of caspase involvement. It will be interesting to study the CaM-FLIP interaction with respect to other death receptors such as DR4, DR5 and TNF-R1, which may provide further insights into the non-apoptotic signaling events mediated by these receptors. Further characterizing the nature of CaM-FLIP interaction may potentially provide new therapeutic targets for cancer therapy.

ACKNOWLEDGMENT

We thank Albert Tousson, Imaging Facility, University of Alabama at Birmingham (UAB), Alabama, for helping us with live cell imaging, Dr. Shi-Yong Sun, Winship Cancer Institute, Emory University, Atlanta, GA, USA for providing with the Lenti-$FLIP_L$ construct. This work was supported by Veterans Affairs Merit Review Award to JMM and JCK, and by research resources of the Genetically Defined Microbe and Expression Core of the UAB mucosal HIV and Immunology Center (R24DK64400).

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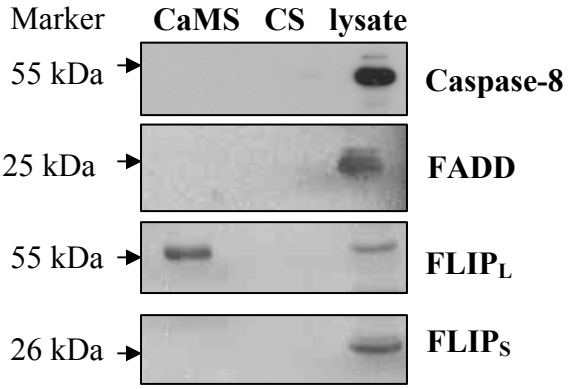
A.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CaMS
CS
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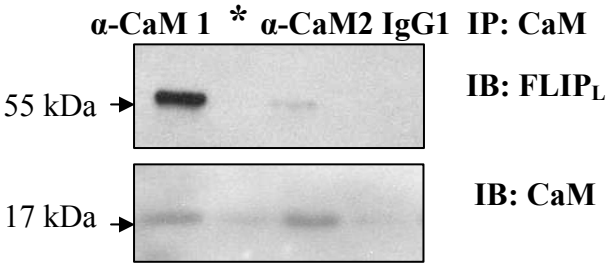
55 kDa
Caspase-8
25 kDa
FADD
55 kDa
FLIPL
26 kDa
FLIPS

B.


α-CaM 1
*
α-CaM2 IgG1
IP: CaM
55 kDa
IB: FLIPL
17 kDa
IB: CaM

C.

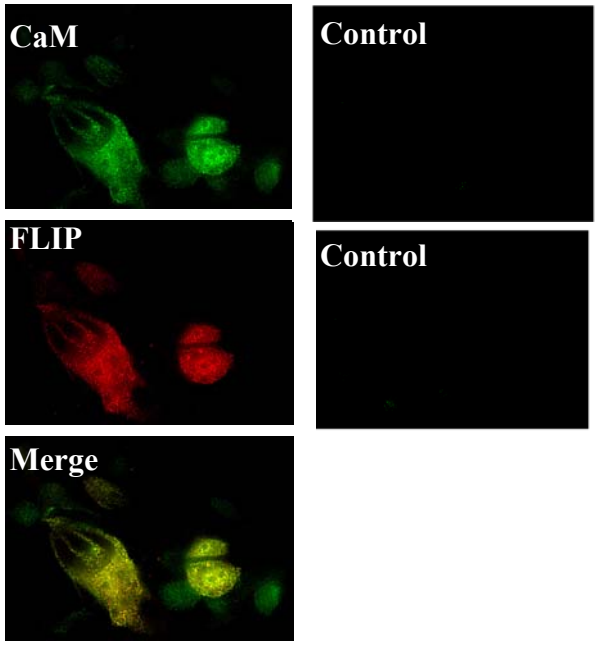

CaM
Control
FLIP
Control
Merge

Figure 1. CaM binding with c-$FLIP_L$

A. CaM binds with c-$FLIP_L$ in cholangiocarcinoma cells. Whole cell lysates from cholangiocarcinoma cells were used for protein pull down assay using Calmodulin-Sepharose (CaMS) or Control Sepharose (CS) beads. The beads were boiled in 2X SDS+10mM EGTA buffer followed by separation with 12% SDS PAGE and immunoblotted for c-FLIP, FADD and caspase-8. The result shown here is representative of 3 independent experiments.

B. CaM and c-$FLIP_L$ were co-immunoprecipitated (Co-IP) by anti-CaM antibody. Cholangiocarcinoma cell lysates were used in co-immunoprecipitation with anti-CaM antibody or isotype matched control mouse IgG1 antibody and immunoblotted for CaM and FLIP as shown in the Fig. 1B**.** α-CaM 1 and α-CaM 2 represent two consecutive samples eluted from α-CaM antibody conjugated column and control IgG1 represents the sample eluted from isotype matched mouse (IgG1) antibody conjugated columns. '*****' in Fig. 1B represents anti-CaM antibody conjugated beads used as a control for heavy chain of the antibody. The data are representative of 4 independent experiments.

C. Immunofluorescence staining showing co-localization of CaM and c-FLIP

Cholangiocarcinoma cells were fixed on the glass cover slips using 3% paraformaldehyde and stained with anti-CaM (Green color) and anti-FLIP (Red color) antibodies. Images were taken at 100X magnification. The merge (Yellow color) represents the picture obtained by merging red and green fluorescent images. 'Antibody control' in the right panel represents Alexa Flour® 488 and Alexa Flour® 594 fluorescent secondary antibodies alone used as negative controls to rule out non specific binding. The data are representative of 5 independent experiments.

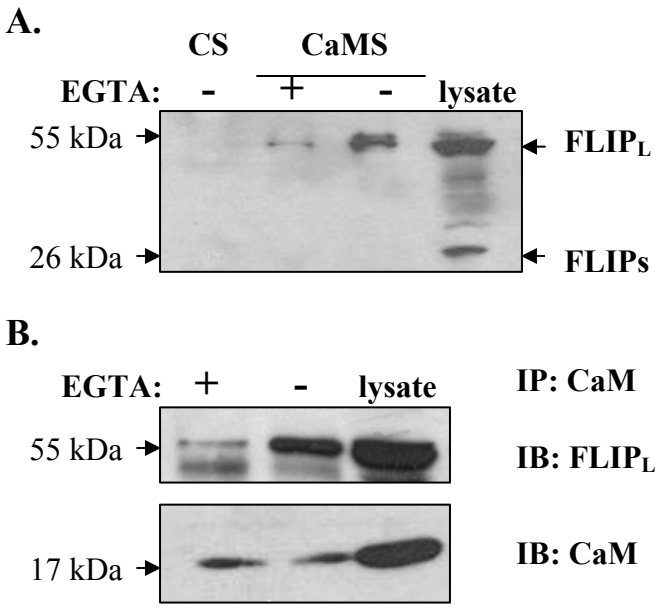
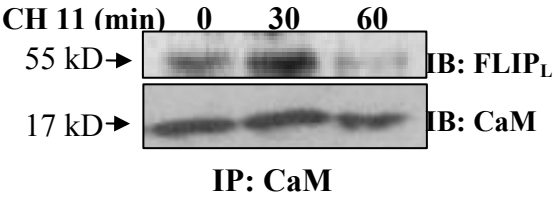


Figure 2. Ca^{++} dependent binding between CaM and c-$FLIP_L$

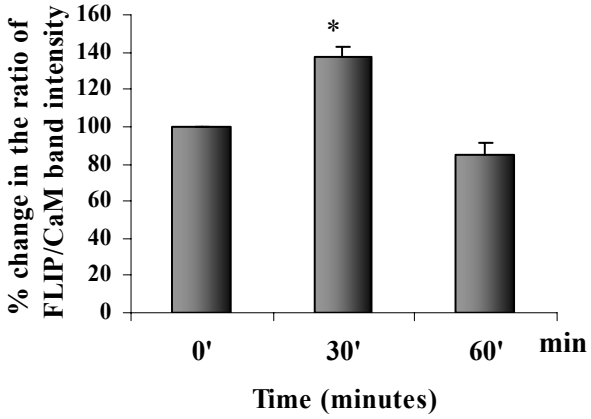
A. Whole cell lysates from cholangiocarcinoma cells were used in protein pull down assay with CaMS or CS beads in the presence or absence of 2mM EGTA. The proteins pulled down were immunoblotted for c-FLIP and CaM. The data are representative of 3 independent experiments.

B. Cholangiocarcinoma cell lysates were used for Co-IP using anti-CaM antibody in the presence (+) or absence (-) of 2mM EGTA and western blots were analyzed for CaM and FLIP. The presence of 2mM EGTA diminished the CaM-$FLIP_L$ binding as decreased $FLIP_L$ co-precipitated in the presence of EGTA. The data are representative of 3 independent experiments.

A.


CH 11 (min) 0 30 60
55 kD
IB: FLIP$_L$
17 kD
IB: CaM
IP: CaM

B.


% change in the ratio of FLIP/CaM band intensity
160
140
120
100
80
60
40
20
0
*
0'
30'
60'
min
Time (minutes)

C.

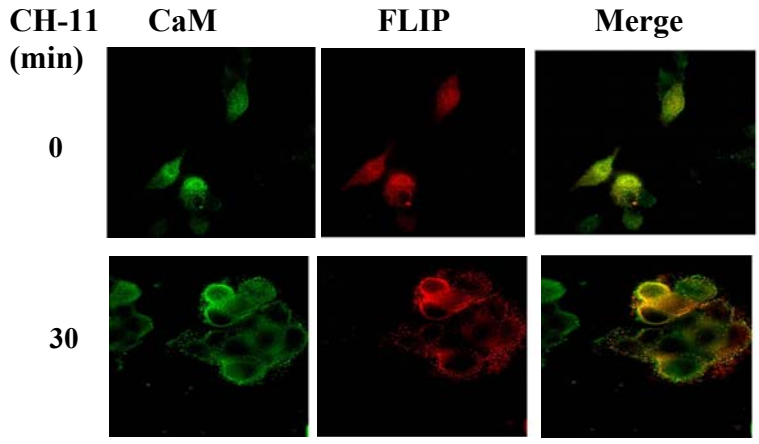

CH-11 (min)
CaM
FLIP
Merge
0
30

Figure 3. Changes in CaM-FLIP interaction in response to Fas activation
A. Cholangiocarcinoma cells were treated with 500ng/ml of Fas activating antibody (CH-11) for 30 and 60 minutes. The whole cell lysates were used for co-immunoprecipitation using anti-CaM antibody and immunoblotted for CaM and c-FLIP. **B.** The band intensities for CaM and FLIP were measured by densitometry. The percentage change in the ratio of FLIP/CaM band intensities, shown in the figure as mean ± SE, were calculated for the indicated times (n=6, p=0.001). **C.** Immunofluorescence staining for CaM (Green) and FLIP (Red) after activation of cholangiocarcinoma cells with CH-11 for 30 minutes showed the movement of CaM and FLIP towards the same sub-cellular compartment as shown in the lower panel in Fig. 3C. The upper panel shown as 0' represents untreated cholangiocarcinoma cells. The data are representative of 3 independent experiments.

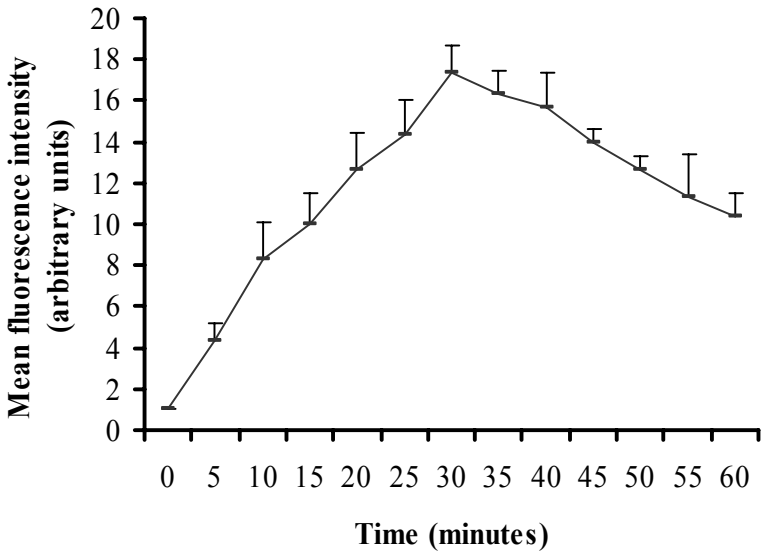


Figure 4. Changes in intracellular Ca^{++} with Fas activation in cholangiocarcinoma cells
The intracellular Ca^{++} was labeled using FLUO-4 AM dye and after recording a baseline image, Fas activating antibody (CH-11, 500ng/ml) was added to the sample and images were recorded at one minute interval over 60 minutes. Isotype matched mouse IgM antibody was added as a negative control that showed no changes in intracellular Ca^{++} and 1uM thapsigargin was added at the end of the experiment as a positive control showing a large increase in intracellular Ca^{++} levels (data not shown). The levels of intracellular Ca^{++} peaked at about 30 minutes and decreased gradually over the next 30 minutes. The data are representative of 3 independent experiments.

Note: From "Calmodulin binding to cellular FLICE like inhibitory protein modulates Fas-induced signaling" by Pritish S Pawar, Keith J Micoli, Haitao Ding, William J Cook, John C Kappes, Yabing Chen and Jay M McDonald, *Biochemical Journal, in press,*

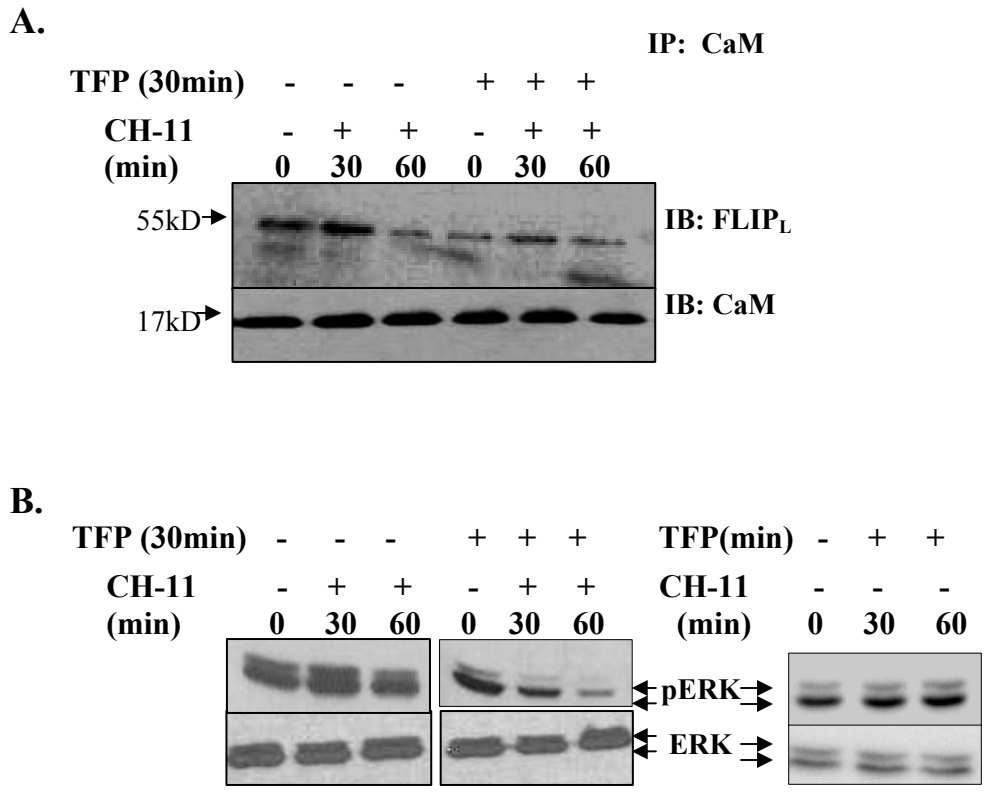


Figure 5. CaM-antagonist, trifluoperazine (TFP), interferes with CaM-FLIP binding with concurrent reduction in phosphorylation of ERK

A. Cholangiocarcinoma cells were treated with 10uM TFP alone or Fas agonist antibody (CH-11) with or without pretreatment for 30 minutes with TFP. The whole cell lysates from these cells were used for co-immunoprecipitation using anti-CaM antibody and immunoblotted for CaM and c-FLIP. **B.** The whole cell lysates from these cells were separated by 12% SDS PAGE and immunoblotted for phosphorylated and total ERK (p44/42). The data are representative of three independent experiments.

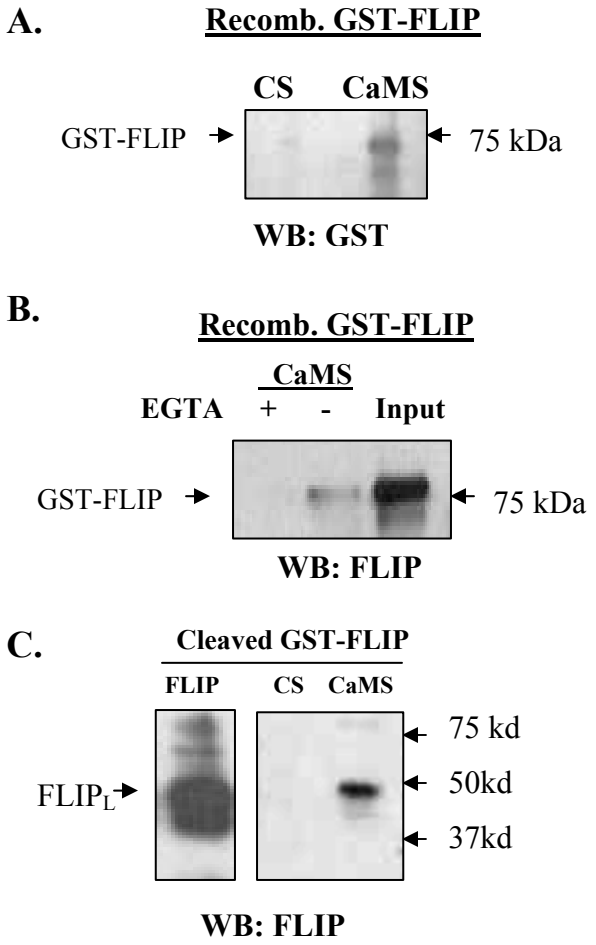
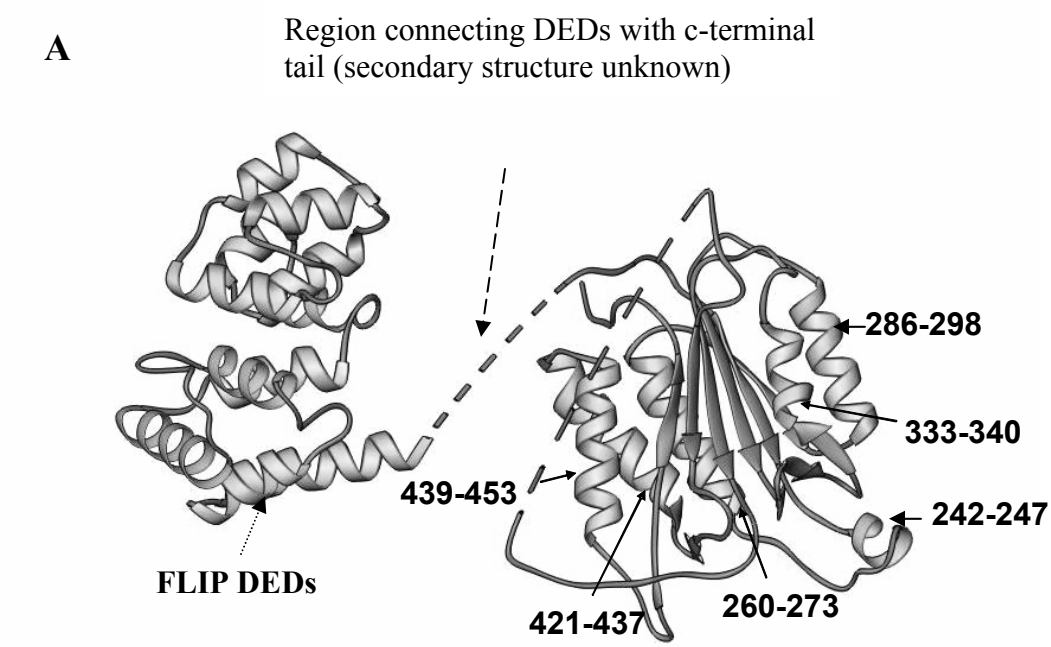


Figure 6. CaM binds directly with recombinant FLIP protein
A. GST-FLIP fusion protein was used for protein pull down using CaMS or CS beads. The proteins obtained from binding reaction were separated by 10% SDS PAGE. Western blot using anti-GST antibody shows that GST-FLIP fusion protein was pulled down specifically by CaMS beads. **B.** Binding between purified GST-FLIP and CaM was diminished in the presence of EGTA as shown from western blot for c-FLIP in Fig. 6B. Input indicates 1/30th of the lysate used in protein pull down with CaMS. **C.** GST tag was cleaved from GST-FLIP fusion protein using Factor Xa and this purified FLIP protein bound with CaMS beads specifically as shown in Fig. 6C. The data shown are representative of 3 independent experiments.


A
Region connecting DEDs with c-terminal tail (secondary structure unknown)
286-298
333-340
439-453
242-247
FLIP DEDs
421-437
260-273

B.

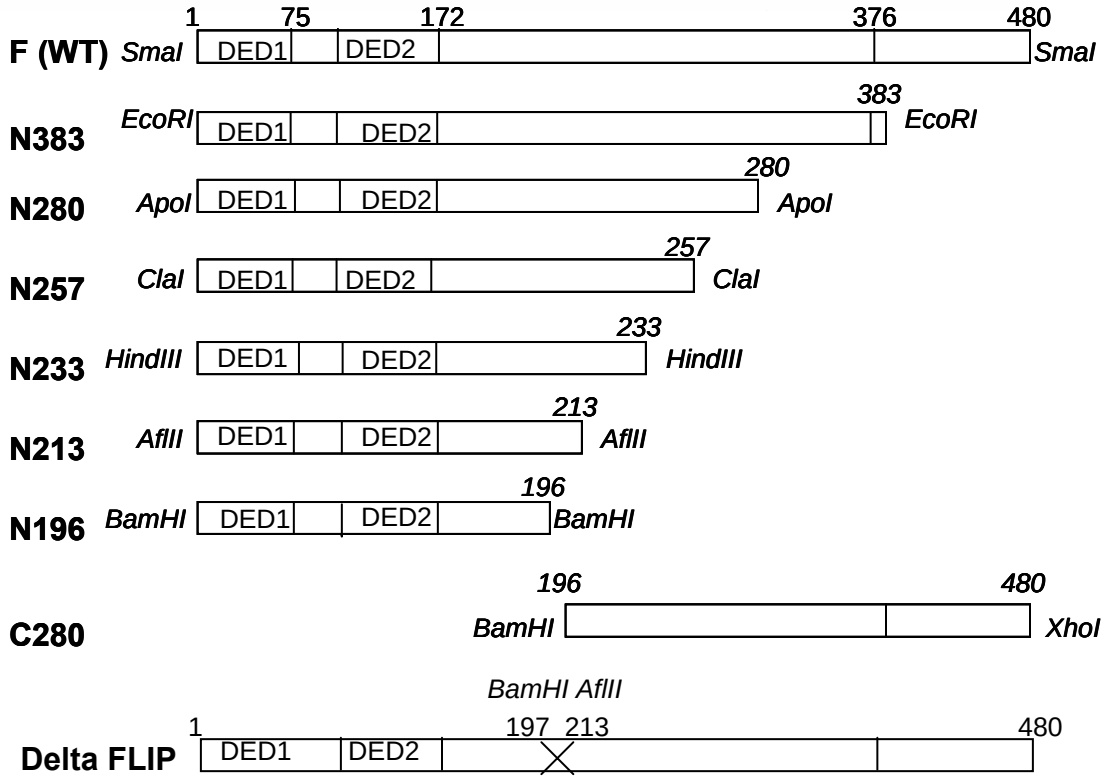

1
75
172
376
480
F (WT)
SmaI
DED1
DED2
SmaI
383
N383
EcoRI
EcoRI
280
N280
ApoI
ApoI
257
N257
ClaI
ClaI
233
N233
HindIII
HindIII
213
N213
AflII
AflII
196
N196
BamHI
BamHI
196
480
C280
BamHI
XhoI
BamHI AflII
1
197
213
480
Delta FLIP

Figure 7. $FLIP_L$ structure and generation of deletion mutants
A. Computer modeling of $FLIP_L$ structure. The presence of helical structures in $FLIP_L$ sequence was determined using caspase-8 homology model. Based on the binding analysis using GST-FLIP deletion mutants, the CaM binding region appears to lie in a region of c-$FLIP_L$ shown by a dotted line. The structure of FLIP DEDs is shown on the left side of this dotted line and on the right side of dotted line is the c-terminal tail of $FLIP_L$, modeled on the basis of sequence homology with caspase-8. **B.** Generation of GST-FLIP deletion mutants. Schematic representation of various GST-FLIP mutants; F (WT): Full length/wild type c-FLIP, N383, N280, N257, N233, N213, N196: mutants with respective numbers of N-terminal amino acids, C280: mutant with C-terminal 280 amino acids, delta FLIP: full length $FLIP_L$ with deletion of amino acids 197-213.

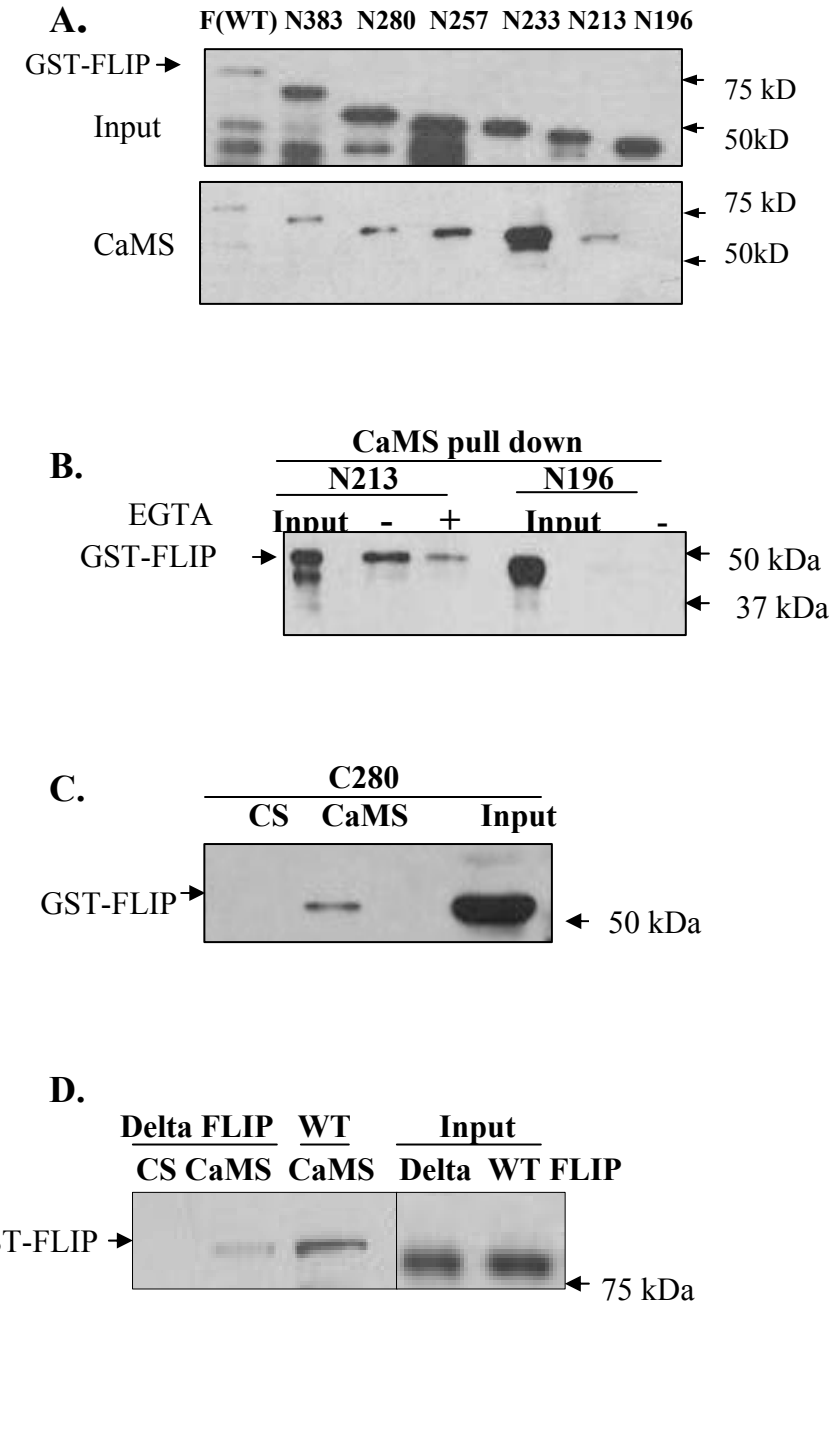

A.
F(WT) N383 N280 N257 N233 N213 N196
GST-FLIP
Input
75 kD
50kD
CaMS
75 kD
50kD
B.
CaMS pull down
N213
N196
EGTA
Input - + Input -
GST-FLIP
50 kDa
37 kDa
C.
C280
CS CaMS Input
GST-FLIP
50 kDa
D.
Delta FLIP WT Input
CS CaMS CaMS Delta WT FLIP
GST-FLIP
75 kDa

Figure 8. Binding of GST-FLIP mutants with CaM

Proteins pull down studies with 20ul 1:1 slurry of CaMS or CS beads were performed using various GST-FLIP mutants. Proteins bound to the CaMS beads were separated by 12% SDS PAGE and western blot analyses were performed using anti-GST and/or anti-FLIP antibodies. **A.** Upper panel in Fig. 8A shows inputs ($1/30^{th}$) of various GST-FLIP mutants. The lower panel shows binding of the mutants with CaMS. Fragments N383, N280, N257, N233 and N213 bound with CaMS but fragment N196 did not bind with CaMS. **B.** The effects of EGTA on the binding of the FLIP fragment N213 and N196. Input indicates $1/30^{th}$ of the GST-FLIP lysates used in protein pull down assay. **C.** CaMS pull down analysis of FLIP fragment C280, which lacks N-terminal region (196 amino acids) containing DEDs. **D.** CaMS pull down analysis of delta FLIP fragment with deletion of amino acids 197-213 from full length $FLIP_L$. Representative blots of 3 experiments are shown.

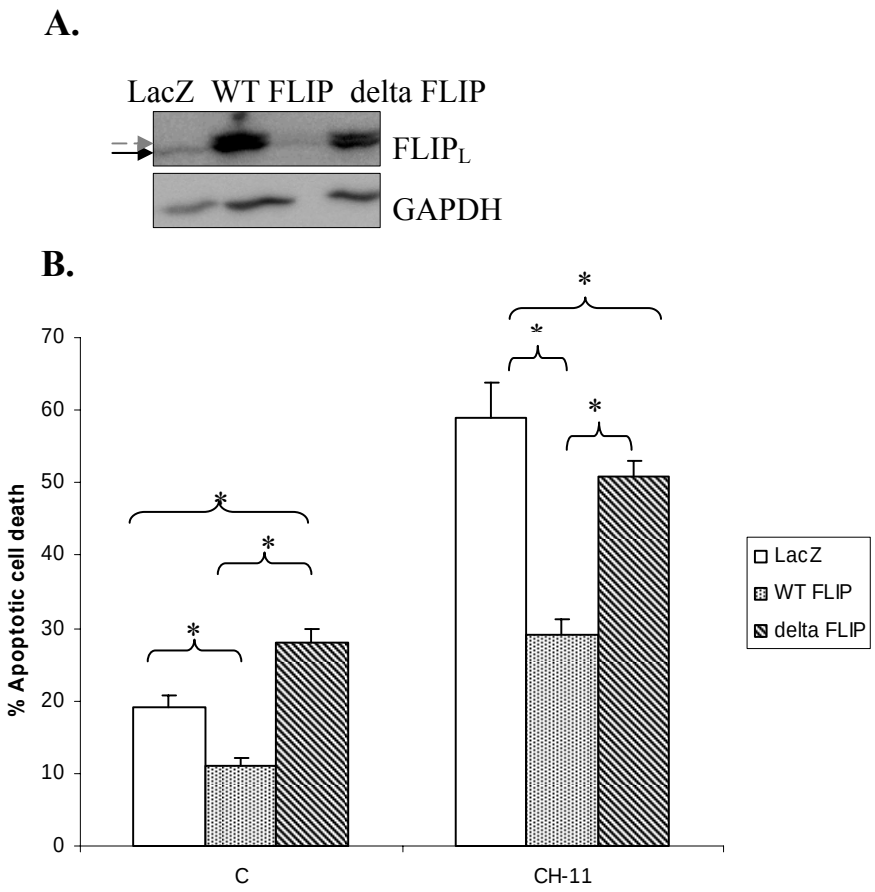


Figure 9. Effect of disrupted CaM-FLIP binding on Fas-mediated apoptosis
A. Western blot analysis for FLIP expression using cell lysates extracted from control cholangiocarcinoma cells (LacZ) or cells over expressing WT and mutant delta FLIP (with deletion of CaM binding region). Native $FLIP_L$ is marked by a bold arrow whereas over expressed FLIP is marked by a dotted grey arrow. **B.** Apoptosis was determined by Annexin V and propidium iodide staining in cholangiocarcinoma cells over expressing WT FLIP, delta FLIP or LacZ as a control. Spontaneous apoptosis in WT FLIP over expressing cells was compared with control vector (LacZ) transduced cells and that of delta FLIP over expressing cells was compared with LacZ as well as WT FLIP over expressing cholangiocarcinoma cells. Fas-induced apoptosis in each cell line was found to be statistically significantly different compared with control conditions in respective cell lines (statistical significance not marked). Results shown as means±SE (n=8). Statistical significance (*) represents $p<0.05$ for comparisons shown by brackets.

MOLECULAR MECHANISMS OF TAMOXIFEN THERAPY FOR CHOLANGIOCARCINOMA: ROLE OF CALMODULIN

by

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Submitted to Clinical Cancer Research

Format adapted for dissertation

ABSTRACT

Cholangiocarcinoma is a highly fatal tumor arising from biliary epithelium with very limited therapeutic options. We previously reported that Calmodulin (CaM) antagonists, tamoxifen (TMX) and trifluoperazine (TFP), induced apoptosis in cholangiocarcinoma cells. In the present studies, we determined the molecular mechanisms responsible for TMX-induced apoptosis in cholangiocarcinoma and further characterized its effects on tumorigenesis. Using a mouse xenograft model, we demonstrated that intra-peritoneal or intra-tumoral injection of TMX reduced cholangiocarcinoma tumorigenesis by 40-80%. In cells isolated from mouse tumor xenografts, TMX inhibited phosphorylation of AKT (pAKT) and c-FLIP expression, both anti-apoptotic molecules interacting with CaM. Immunohistochemical analyses of the tumors further demonstrated that pAKT was identified in all non-treated tumors (pAKT-positive tumor cells=27.±11%) but was absent in TMX-treated tumors. In-vitro, TMX activated caspase-8 and -10, and their respective inhibitors partially blocked TMX-induced apoptosis by 43% and 36% respectively ($n=3$, $p\leq0.05$). Additionally, over-expression of c-FLIP inhibited TMX-induced apoptosis and enhanced tumorigenesis of cholangiocarcinoma cells in nude mice whereas deletion of the CaM binding domain of over-expressed c-FLIP restored the sensitivity to TMX and inhibited tumorigenesis. Thus TMX-induced apoptosis of cholangiocarcinoma cells is partially dependent on caspases, inhibition of AKT phosphorylation and FLIP expression. Further, CaM-FLIP binding

appears to be important in mediating the anti-apoptotic effects of c-FLIP. The present studies therefore support the concept that TMX may be used effectively as a therapy for cholangiocarcinoma and possibly other malignancies, in which the CaM targets, AKT and c-FLIP, play a role in pathogenesis.

Key words: Tamoxifen (TMX), Calmodulin (CaM), Cholangiocarcinoma, cellular, FLICE like inhibitory protein (c-FLIP), AKT

INTRODUCTION

Cholangiocarcinoma is a highly malignant neoplasm originating from cholangiocytes of the intra- and/or extra-hepatic biliary system. Extensive surgical resection is the only effective therapy available for long term survival as conventional chemotherapy and radiotherapy are generally ineffective. The overall survival following diagnosis of unresectable cholangiocarcinoma is less than 12 months [1]. The 5 year survival for the patients undergoing surgical resection ranges from 0-40% [2]. Over the last few years there has been a marked increase in the incidence and mortality from cholangiocarcinoma warranting an increasing need for effective strategies to prevent and treat this lethal tumor [3]. In search of an efficient chemotherapy for cholangiocarcinoma, we identified that tamoxifen (TMX), a potent calmodulin (CaM) antagonist [4, 5] induces apoptosis in cholangiocarcinoma cells in-vitro and inhibits tumorigenesis in vivo in nude mice xenografts [6]. We demonstrated that the apoptosis-inducing abilities of tamoxifen in cholangiocarcinoma cells appear to be associated with its role as a CaM-antagonist [7]. In the present studies, we further determined the molecular mechanisms responsible for

tamoxifen-induced apoptosis in cholangiocarcinoma and its therapeutic potential in nude mice xenografts.

TMX was first identified as an anti fertility drug in the early 1960s. It was approved in UK, for the first time, as an anti-cancer agent [8]. Today it is one of the most widely used anti-cancer drugs worldwide, is relatively cheap and has few side effects [9, 10]. Over the last two decades, substantial effort has been focused on characterizing the molecular mechanisms behind the effects of TMX, including TMX-induced apoptosis. Various pathways such as protein kinase C, TGF-β, AKT, MAPK, c-myc and calmodulin have been implicated in TMX-induced effects on human cancer cell lines [11]. Further, now it is clear that TMX can activate genomic (estrogen receptor-mediated) as well as non genomic (estrogen receptor-independent) pathways in dose-dependent and cell type specific manners [4, 11, 12]. Our laboratory has been focusing on the CaM-dependent aspect of TMX biology in a cholangiocarcinoma tumor model using a human cholangiocarcinoma cell line that lacks estrogen receptors [7]. We have reported previously that CaM-antagonists, TMX and trifluoperazine (TFP), induced apoptosis in cholangiocarcinoma cells in a Fas-related manner [7, 13]. Therefore, in this report, we further characterized the modulation of CaM signaling by TMX in both, cell lines in vitro and a cholangiocarcinoma xenograft model in nude mice.

CaM is an important Ca^{++} binding protein that has been well conserved through evolution and mediates various signaling pathways induced by Ca^{++} [14, 15]. CaM interacts with a variety of proteins in Ca^{++} dependent as well as independent manners [16]. With increases in local Ca^{++} concentration, it undergoes a conformational change and interacts with its target proteins such as CaMKII, calcineurin and nitric oxide

synthase to mediate various downstream Ca^{++} signaling events [17, 18]. Increase in intracellular Ca^{++} is shown to be important in cellular proliferation as well as apoptosis and CaM is believed to be important in mediating these signaling events [14, 19]. Amongst the wide variety of proteins interacting with CaM, the two proteins which we identified to be affected by TMX in cholangiocarcinoma tumor model are pro-survival molecules, AKT and cellular FLICE like inhibitory protein (c-FLIP).

c-FLIP is largely described as an anti-apoptotic protein that interferes with activation of initiator caspases, thus inhibiting the downstream apoptotic signaling [20, 21]. It is over expressed in various cancer cell lines and is associated with resistance to chemotherapeutic agents [22-24]. Recently we identified a direct Ca^{++} dependent interaction between CaM and FLIP which is regulated by changes in intracellular Ca^{++} levels [25]. AKT, also called protein kinase B, is another important pro-survival molecule that has been shown to interact with CaM, possibly facilitating its translocation to the plasma membrane [26]. Classically, AKT signaling is initiated by binding of a growth factor to a receptor tyrosine kinase, leading to activation of phosphatidylinositol-3-kinase (PI3K) [27-29]. Activated PI3K leads to the generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3) which then interacts with AKT and anchors the AKT kinase to the plasma membrane where it undergoes phosphorylation and activation by various kinases [30, 31]. Thus translocation of AKT to the plasma membrane is an important step in its activation. CaM was shown to compete with PIP_3 for binding with AKT in a Ca^{++} dependent manner such that elevations in Ca^{++} concentrations disrupted the interaction between AKT and phosphoinositides [26].

In the investigations presented here using cholangiocarcinoma as a model, we demonstrate that TMX, a potent CaM antagonist, inhibits phosphorylation of AKT and expression of c-FLIP, both of which are CaM binding proteins. Furthermore TMX is effective in decreasing tumor size in a mouse xenografts model probably by modulating CaM signaling pathways.

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). Stable cell lines over expressing wild type and delta FLIP proteins were generated using lentiviral $FLIP_L$ expression vector as described previously [25]. Delta FLIP protein was generated by deleting CaM binding region (aa 197-213) from $FLIP_L$ as decribed previously [25]. 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-FLIP, NF6 (Alexis Corp, CA), anti-caspase-8, anti-caspase-10, ant-caspase-3, ant-caspase-9, ant-AKT, anti-phospho-AKT (Cell Signaling, Boston, MA), anti-GAPDH (Research Diag. Inc, Concord, MA). The monoclonal antibody to CaM was developed as described [32] . Goat anti mouse and bovine anti rabbit HRP conjugated antibodies (Santa Cruz Biotech, CA).

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) female Balb/c mice (Charles River Laboratories, MA) were used for tumor inoculation. Briefly, cholangiocarcinoma cells (5×10^6 cells in 200µl PBS/site) were inoculated subcutaneously into the flanks of mice. Tamoxifen (TMX) was purchased from Calbiochem, CA. After one week, mice were randomly assigned into two groups. Tumors in one group were treated with TMX (15µM in 100ul volume per tumor site) injections intra-tumorally and tumors in the other group were injected with PBS as control. For intraperitoneal injections, 0.1mg TMX in 0.1ml oil was administered intra-peritoneally for 3 consecutive days followed by one day of rest for two such cycles. Tumors were examined and measured every 3-4 days using the formula Volume= Length x $\text{Width}^2/2$.

Culture of xenograft tumor-derived cells

Tumors were removed from mice aseptically, minced into pieces and placed in growth media containing collagenase I for 30 min at 37°C followed by mashing through a cell strainer (100 µm, BD Pharmingen). The cell suspension was washed twice with PBS and cultured in RPMI-1640 medium.

Immunohistochemical analysis

Tumors were placed in buffered formalin and embedded in paraffin. Slides were deparaffinized, rehydrated, and heated in 10 mmol/L citrate buffer (pH 6.0) for 40

minutes using a steamer. The slides were blocked with 10% normal rabbit serum for 30 minutes, washed with PBS and incubated with anti-phosporylated-AKT-1 antibody (Santa Cruz Biotech, CA) for 1 hour at room temperature. The secondary biotinylated anti-rabbit IgG was applied for 30 minutes followed by 30 minutes of incubation with a polymer (Envision plus, DAKO, Carpenteria, CA). Slides used as negative controls did not receive the incubation with primary antibodies. After rinsing, slides were treated with diaminobenzidine chromogen solution and counterstained with routine hematoxylin and eosin. Brown staining in greater than 10% of the cells was considered as a positive stain.

Assessment of apoptosis

Annexin V-fluorescein isothiocyanate and propidium iodide (PI) staining was performed using apoptosis detection kit (BD Biosciences, CA) and analyzed by flow cytometry. For studies involving caspase inhibitors, cells were pretreated with caspase inhibitor for 3hrs before addition of TMX. The percentage of cells that are Annexin V positive and PI negative were considered to be apoptotic cells.

Western blot analysis

Protein extracts were isolated from cells using lysis buffer containing 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 1% triton X, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1mM sodium orthovanadate, 1 mM β-glycerophosphate and protease inhibitor cocktail tablets (Roche, Indianapolis, IN). Concentrations of protein were determined with the Bicinchoninic acid kit (Sigma). Proteins were separated by sodium dodecyl sulfate poly-

acrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane as described previously [33]. For CaM, the membranes were fixed in 0.2% glutaraldehyde in tris buffered saline for 30 minutes prior to blocking step. Membranes were incubated with primary antibodies overnight at 4ºC. Horseradish peroxidase (HRP) conjugated secondary antibodies in the blocking buffer were incubated for 2 hours at room temperature. Signals were detected using immobilon western chemiluminescent HRP substrate (Millipore, Billerica, MA) detection kit.

Cell proliferation assay

Proliferation of cholangiocarcinoma cells was analyzed using Thiazolyl Blue Tetrazolium Bromide (MTT) reagent (Sigma Aldrich, MO) as per the manufacturer's recommendations. Briefly, 10000 cells were seeded in 200ul of complete RPMI 1640 medium per well in a 96 well plate. MTT was added on days 0 (measured after allowing the attachment of cells overnight), 2 and 4 to the final concentration of 0.5mg/ml and incubated at 37ºC for 2 hours. The medium was removed and the crystals were dissolved in DMSO followed by measuring the absorbance at 570nm.

Statistical analysis

Results are expressed as means ± SE. Differences between two groups were identified with Student's t-tests. Significance was defined as $p < 0.05$.

RESULTS

TMX inhibited tumorigenesis of cholangiocarcinoma cells and phosphorylation of AKT in nude mice xenografts

We have reported that CaM-antagonists, TMX and TFP, induce apoptosis in human cholangiocarcinoma cells in vitro in a Fas-related manner and probably through CaM-dependent pathways [7]. Further, in order to demonstrate its efficacy in vivo, we showed that intra-peritoneal injections of TMX (0.1mg/mouse) in nude mice bearing cholangiocarcinoma xenografts reduced tumor size by 82% after one week as shown in Fig 1A (tumor volume (mm3), control= 208 ± 27 , TMX= 37 ± 5, n=6, $p<0.0005$). Similar studies performed with intraperitoneal TMX injections over six weeks consistently showed that the cholangiocarcinoma xenografts were significantly reduced in size with TMX treatment [6]. To further characterize the TMX effects, mice bearing tumor xenografts were assigned randomly to two groups for daily intra-tumoral injection with either TMX (100 ul of 15uM stock) or PBS as a control. The tumor volumes were measured every 3-4 days and mice were sacrificed after four weeks of treatment. As shown in Fig. 1B, TMX inhibited tumor growth, resulting in a significant decrease in tumor size as compared to control group (at the end point, control=523±58mm3 and TMX=312±22mm3, n=10, p=0.009). Insert in Fig. 1B shows a representative photograph of the cholangiocarcinoma xenograft growth with PBS (control) and TMX treatment after four weeks. Thus both intra-peritoneal as well as intra-tumoral injections of TMX were

found to be effective in reducing cholangiocarcinoma xenografts growth in nude mice model.

We have previously demonstrated that AKT signaling plays an important role in cholangiocarcinoma pathogenesis [33]. Therefore we characterized the effects of TMX on phosphorylation of AKT in xenografts that were treated with TMX or PBS. As shown in Fig 1C, a and 1C, b, H&E stain for control and TMX treated tumors show the tumor cells arranged in a glandular pattern. The cells in the control section are dysplastic, showing enlarged nuclei with altered nucleo-cytoplasmic ratio and demonstrated prominent nucleoli. The TMX treated cells appear slightly less dysplastic. Previously, we reported that phosphorylation of AKT was increased in human cholangiocarcinoma tumors but not in adjacent non neoplastic bile duct epithelium [33]. Consistently, the immunohistochemical analysis of mice xenografts showed that pAKT, seen predominantly in the cytoplasm as brown stain, was detected in PBS treated tumors (Fig. 1C, c) but not in TMX treated tumors (Fig. 1C, d). Further, 27±11% cells were found to stain positive for pAKT in PBS treated group whereas TMX treated tumors did not show any staining for pAKT (n=7). Thus TMX inhibited phosphorylation of AKT in cholangiocarcinoma xenografts in nude mice.

TMX inhibited phosphorylation of AKT and FLIP expression in tumor cells from mice cholangiocarcinoma xenografts

Cholangiocarcinoma cells were isolated from mice xenografts. TMX induced 1.9 fold increased apoptosis in these cells as compared to control (Fig. 2A). Further western blot analysis of lysates from these cells confirmed that TMX inhibited phosphorylation of AKT and expression of FLIP in these cells but the expression of CaM and Fas was

unaffected by TMX as shown in Fig. 2B. Thus inhibition of pAKT was observed in vitro as well as in vivo in cholangiocarcinoma xenografts in nude mice.

TMX mediated apoptosis involves activation of caspase-10, -8, - 9 and -3 and inhibition of FLIP expression in cholangiocarcinoma cells

We further determined the involvement of caspases, including caspase-10, -8, -3 and -9, in TMX induced apoptosis. As seen in Fig 3A, TMX induced 3.9 fold increase in apoptosis in vitro in cholangiocarcinoma cells as compared to control. It activated caspase-10 and -8, both of which are initiator caspases as seen in Fig. 3B. Further, consistent with our previous reports, TMX activated caspase-9 and -3 as well [13]. Furthermore, we identified that an anti-apoptotic protein, c-FLIP, was down regulated by TMX.

Caspase inhibitors partially blocked TMX induced apoptosis

To determine the requirement of caspase-8 and -10 in TMX induced apoptosis, cholangiocarcinoma cells were pretreated with the respective caspase inhibitors for 3 hrs followed by TMX treatment for 24 hrs. As shown in Fig. 4A and C, TMX-induced apoptosis was partially blocked by both, caspase-8 (43±2%) and -10 (36±5%) inhibitors. Further, caspase-3 activation was blocked by both of these inhibitors, suggesting that both caspase-8 as well as -10 are required for TMX-induced caspase-3 activation in cholangiocarcinoma cells. Western blot analysis, illustrated in the right side panels of Fig. 4B and D, show that caspase -8 and -10 inhibitors effectively blocked TMX induced activation of the respective caspases. Further, we confirmed that the pan caspase inhibitor, zVAD, also caused partial inhibition of TMX induced apoptosis as shown

previously [13], suggesting that TMX induced apoptosis is only partially caspase dependent. However, the caspase inhibitors may not be entirely specific and the specificity can be diminished with increased concentrations and prolonged incubation times [34, 35]. Nevertheless, it can be concluded from these studies using caspase-inhibitors that TMX induces apoptosis in caspase dependent as well as independent manners.

FLIP over expression increases the tumorigenicity and decreases the sensitivity of cholangiocarcinoma cells to TMX induced apoptosis: restoration by deletion of CaM-binding region from c-FLIP

FLIP interferes with the activation of initiator caspases and thus blocks the apoptotic signaling [36, 37]. Increased expression of FLIP has been identified in numerous cancer cell lines and associated with resistant phenotypes of various cancers including cholangiocarcinoma [38] [39-41]. Recently we reported that CaM interacts directly in a Ca^{++} dependent manner with c-$FLIP_L$. We found that TMX inhibited activation of AKT as well as FLIP expression (Fig. 2 and 3), both of which are known to be CaM binding proteins [25, 26]**.** Thus we asked two questions: 1. Does FLIP expression modulate the apoptotic response to TMX? and 2. If so, is it dependent on FLIP binding with CaM? Cholangiocarcinoma cells stably over-expressing wild type (WT) $FLIP_L$, delta $FLIP_L$ (mutant with deletion of CaM binding domain) or LacZ (control) proteins were generated as described previously [25] and treated with TMX for 24 hrs followed by assessment of apoptotic cell death by Annexin/PI staining. As shown in Fig. 5A, FLIP over expression decreased the sensitivity of cholangiocarcinoma cells to TMX, which was restored by deletion of CaM binding domain from over-expressed

$FLIP_L$. The effects of WT and delta FLIP over expression on TMX-induced apoptosis were confirmed by using another known CaM-antagonist, W-7. Thus WT FLIP over expression decreased W-7-induced apoptosis which was restored after deletion of the CaM binding region from $FLIP_L$, confirming that TMX induced apoptosis involves modulation of CaM signaling in cholangiocarcinoma (Fig 5B). Further, as shown in Fig. 5C, disruption of CaM-FLIP binding also resulted in decreased cell proliferation as compared to WT FLIP expressing cholangiocarcinoma cells in which CaM-FLIP binding is intact. Thus over expression of $FLIP_L$ appears to be an important regulator of TMX induced apoptosis and CaM binding to $FLIP_L$ is critical for mediating the anti-apoptotic/pro-survival effects of FLIP.

The tumorigenicity of WT and delta FLIP over expressing cholangiocarcinoma cells was analyzed in nude mice to determine if $FLIP_L$ expression and disruption of CaM-$FLIP_L$ binding would affect tumorigenesis of cholangiocarcinoma xenografts in nude mice. Thus $2x10^6$ cells [LacZ , WT FLIP and delta-FLIP expressing cholangiocarcinoma cells] were injected in the flanks of 6-7 weeks old athymic nude mice. The tumor volumes were measured every 3-4 days for 18 days. As shown in Fig. 5D, at day 7, no significant differences were observed in tumorigenesis of LacZ, WT and delta FLIP cells. WT FLIP cells formed significantly bigger tumors than control LacZ and delta-FLIP cells whereas delta-FLIP cells formed the smallest tumors at day 18 (tumor volumes in mm^3, control LacZ = 124±12, WT FLIP = 170±25, delta FLIP = 82±15). At day 14, delta FLIP cells grew significantly smaller tumors than both LacZ and WT FLIP cells (LacZ = 99±9, WT FLIP =101±16, delta FLIP = 62±11), supporting the concept that the interaction between CaM and FLIP is important in cholangiocarcinoma pathogenesis.

DISCUSSION

TMX is an extensively tested and widely used drug worldwide. It is inexpensive and has a low side effect profile, thus providing a very attractive therapeutic option [9, 10]. Cholangiocarcinoma, a highly lethal tumor arising from biliary epithelium, has a poor 5 year survival rate and no effective therapy [1, 2]. With increasing incidence of this disease, it is imperative that novel strategies are identified to prevent and treat this deadly disease. Our group previously showed that TMX induces apoptosis in cholangiocarcinoma cells, both in vitro and in vivo in a mouse xenograft model [6, 7, 13]. Further, along with TMX which is a potent CaM-antagonist, we demonstrated that other CaM-antagonists, W-7 and TFP, also induced apoptosis in human cholangiocarcinoma cells [7]. In addition, the apoptosis inducing abilities of CaM-antagonists were found to correlate with the sensitivity of cholangiocarcinoma cells to Fas mediated apoptosis. Thus there appears to be a cross talk between Fas and CaM pathways which was further substantiated by demonstrating a direct binding between CaM and Fas receptor [42].

The apoptosis inducing ability of TMX in human cholangiocarcinoma cell lines appear to be estrogen receptor independent as these cells lack estrogen receptors [7]. We therefore proposed that TMX induced apoptosis in cholangiocarcinoma cells by modulating CaM signaling. CaM is an important Ca^{++} binding protein that binds with a variety of proteins in response to increases in intracellular Ca^{++} and mediates a variety of signaling pathways ranging from proliferation, differentiation and apoptosis [17, 18]. In

neocortical cell cultures, Ca^{++}-CaM was shown to be important in mediating cell survival signals of the brain-derived neurotrophic factor by affecting PI3K-AKT pathway [43]. Mutants of CaM with disabled Ca^{++} binding domains and CaM-antagonists were shown to inhibit activation of AKT and significantly decreased long term cell survival, suggesting a pivotal role for CaM in AKT mediated cell survival. Furthermore, recently CaM has been shown to interact with AKT and the interaction is believed to modulate the membrane translocation of this kinase by interfering with the AKT-phosphoinositides binding that is important for membrane translocation and subsequent phosphorylation of AKT [26]. Thus CaM appears to modulate AKT signaling pathway via its direct interaction with AKT. Consistently, in our studies, we have shown that the CaM-antagonist, TMX, inhibited the phosphorylation of AKT and resulted in increased apoptotic cell death. We also showed that TMX down regulated the expression of an anti-apoptotic molecule, c-FLIP. Interestingly, FLIP is a CaM-binding protein, binding with CaM directly in a Ca^{++} dependent manner [25]. Further, inhibition of pAKT has also been associated with decreased expression of c-FLIP in various tumors but the mechanism is not clearly understood [33, 44]. It not known whether TMX-mediated decreased expression of c-FLIP observed concurrently with inhibition of pAKT is regulated at transcriptional level or at the protein level through proteosomal degradation. Nevertheless with TMX affecting AKT and FLIP, both CaM binding proteins, the apoptosis-inducing ability of TMX is likely to be related to the modulation of CaM signaling pathways.

AKT is a pro-survival protein, and it is up regulated in several tumors [45, 46]. We recently demonstrated that AKT is activated in cholangiocarcinoma cells that are resistant to Fas–induced apoptosis and inhibition of AKT using dominant negative AKT

enhances the sensitivity of these cells to Fas mediated apoptosis [33]. Further inhibition of pAKT has been linked to FLIP down regulation in several cancer cell lines [33, 44]. FLIP is an anti-apoptotic protein that abrogates apoptotic signaling upstream in the pathway. Thus it could provide an efficient therapeutic target. Further, we demonstrated that FLIP expression is an important determinant of the response to TMX as FLIP over expression reduced the sensitivity of cholangiocarcinoma cells to TMX. Thus, our studies, for the first time, provide evidence that FLIP expression could be an important determinant of the response to chemotherapy in cholangiocarcinoma. Since TMX mediated inhibition of AKT and FLIP is related to the CaM signaling, the next logical step would be separately modulating AKT, CaM or FLIP and assessing responses to TMX. Since AKT and CaM are involved in the regulation of a variety of signaling pathways and cell processes, we decided to target FLIP, specifically the CaM-FLIP binding, to assess its effect on TMX mediated apoptosis. The expression of WT FLIP in cholangiocarcinoma cells decreased their sensitivity to TMX-induced apoptosis, but when CaM-FLIP binding was compromised by over expressing a FLIP mutant that lacked CaM binding region, the sensitivity to TMX was restored. The results were confirmed with another CaM-antagonist, W-7, suggesting the involvement of CaM in FLIP mediated resistance to apoptosis. Further, disrupted CaM-FLIP binding decreased cell proliferation and decreased tumorigenicity in nude mice as compared to WT FLIP over expressing cells with intact CaM-FLIP binding. Thus CaM-FLIP binding appears to be important in mediating pro-survival effects of c-FLIP and exerting resistance to TMX in FLIP over expressing cholangiocarcinoma cells. Thus TMX alone or coupled with a drug specifically targeting CaM-FLIP binding could provide an effective combination

therapy for lethal tumors in which FLIP expression is known to impair response to therapy.

With a variety of signaling pathways modulated by CaM and its recently described cross talk with the Fas pathway, it appeared to provide a potential therapeutic target for cholangiocarcinoma. Interestingly, CaM binding with both Fas and FLIP is Ca^{++} dependent but their CaM binding sites are structurally different. While CaM binding with Fas is mediated via a classic 1-5-10 CaM binding motif [42], its binding with FLIP is mediated by a non-classic motif [25]. Thus there may be differences in affinity between CaM-Fas and CaM-FLIP binding in basal and activated states. CaM binding with these proteins may confer structural stability and/or conformational changes required for downstream signaling events. It is likely that targeting each of these specific interactions could provide efficient ways to modulate apoptotic sensitivity of cancer cells and novel targets in therapy.

We propose a model for TMX induced apoptosis in cholangiocarcinoma cells operating through CaM. As shown in Fig.6, TMX induces activation of caspase-8 and -10 leading to activation of caspase-3 and at the same time it also inhibits pro-survival signals by inhibiting phosphorylation of AKT and down regulating FLIP expression. Importantly, CaM has been shown to interact directly with Fas and thus the Fas-CaM interaction may compete with Fas-FADD interaction to interfere with the death inducing signaling complex (DISC) assembly. Furthermore, CaM-FLIP binding adds another arm to this inhibitory effect of CaM on Fas induced apoptosis by allowing increased FLIP recruitment into the DISC that further interferes with caspase activation. Thus TMX, by virtue of its anti-CaM properties, may be able to diminish the inhibitory effects of CaM

on Fas-induced apoptosis and regulates the CaM signaling pathway via modulation of c-FLIP and AKT signaling to induce apoptosis and inhibit tumorigenesis in cholangiocarcinoma.

ACKNOWLEDGEMENT

This work is supported by VA merit review award to JMM. We thank Marion Spell, Center for AIDS Research, University of Alabama at Birmingham, for technical assistance with flow cytometry.

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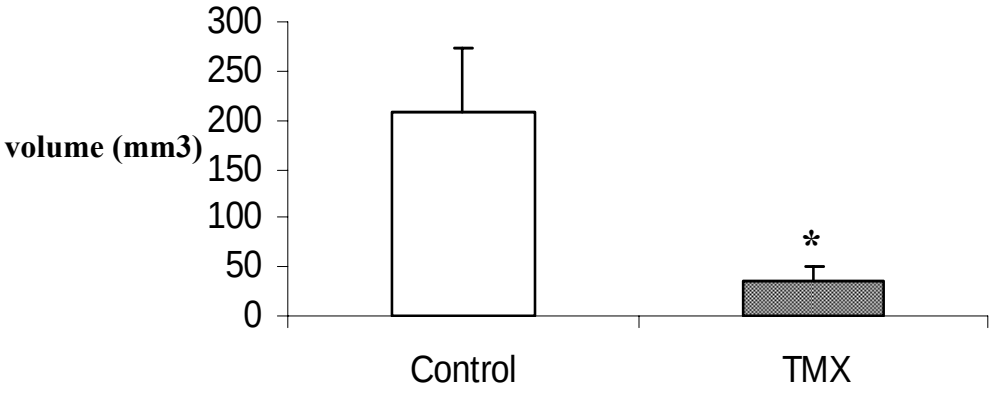
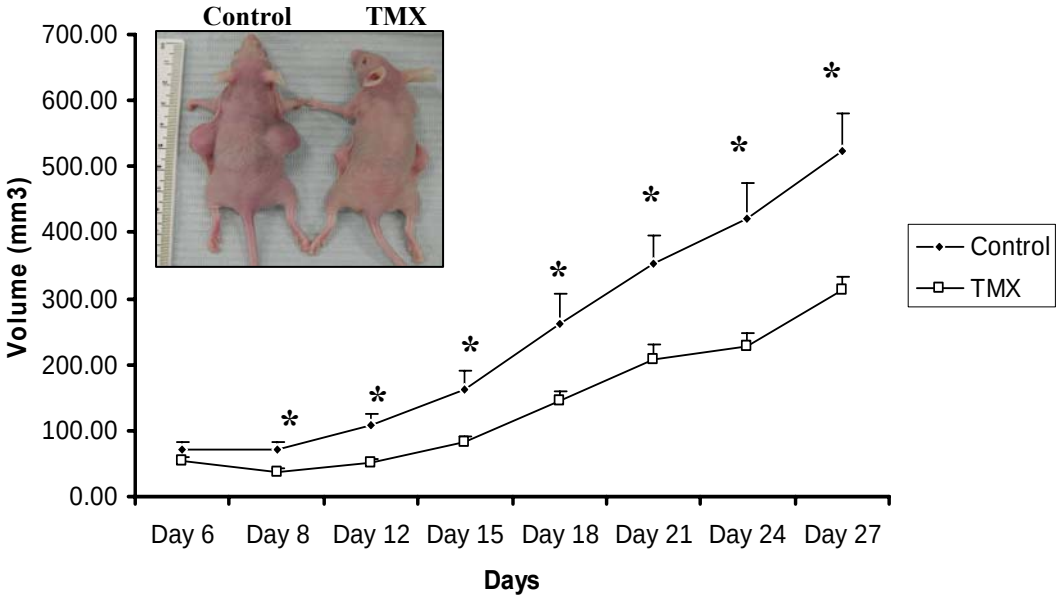
A.


volume (mm3)
300
250
200
150
100
50
0
*
Control
TMX

B.


Control
TMX
Volume (mm3)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Day 6
Day 8
Day 12
Day 15
Day 18
Day 21
Day 24
Day 27
Days
Control
TMX

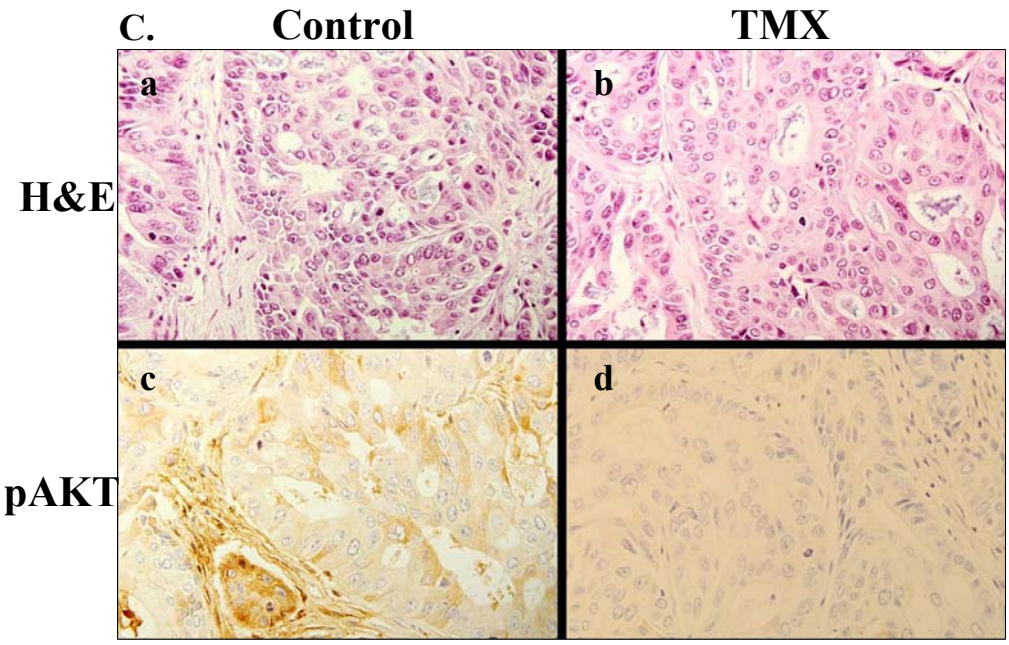


Figure 1. Tamoxifen inhibits cholangiocarcinoma tumorigenesis and phosphorylation of AKT in nude mice.

Cholangiocarcinoma cells were inoculated subcutaneously into the flanks of eight-week old athymic (nu/nu) female Balb/c mice. After 6 days, mice were randomly assigned into two groups. **A.** 0.1ml oil as a control or 0.1mg TMX in 0.1ml oil was administered intra-peritoneally in each mouse daily as described in Materials and Methods and tumors were measured after one week of treatment. The data represents mean±SE (n=6, $p<0.05$). **B.** Tumors in one group were injected with Tamoxifen (100ul TMX stock of 15μM) and tumors in the other group were injected with PBS as control. **A.** The data represents mean tumor volume±SE (n=10, '*' indicates $p<0.05$). Insert in **Fig 1B**. shows a representative picture of tumor-bearing mice in the control and TMX-treated groups. **C.** Hematoxylin and Eosin (H&E) staining of a section from a PBS (Fig. 1C, a) and a TMX-treated tumor (Fig. 1C, b), immunohistochemical staining of pAKT on a PBS (Fig. 1C, c) and a TMX-treated tumor section (Fig. 1C, d) (magnification: 400X).

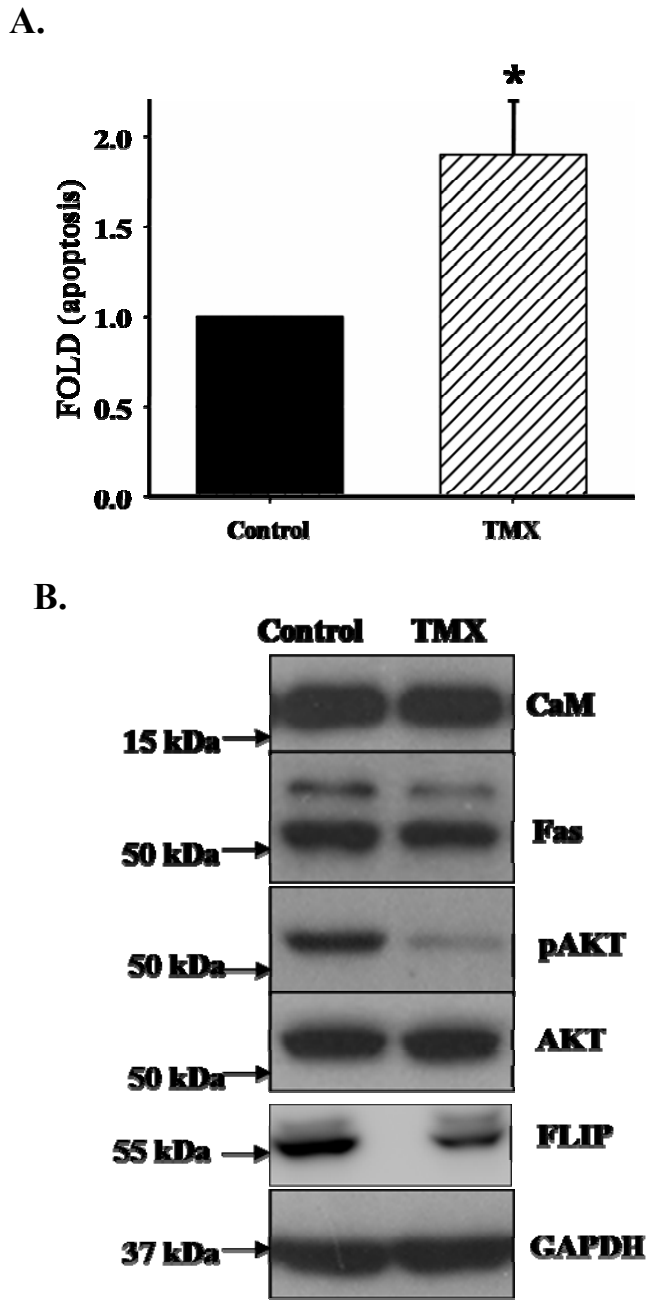
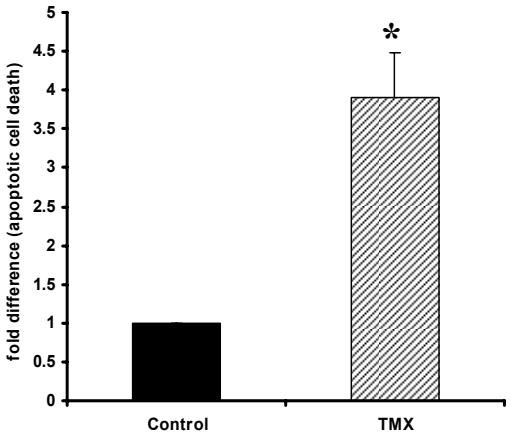


Figure 2. Apoptosis in response to TMX in tumor cells isolated from cholangiocarcinoma xenografts from nude mice.
Cells from cholangiocarcinoma xenografts were treated with or without TMX (10μM) for 24 hours. **A.** Apoptosis was determined by AnnexinV/PI staining and results shown are mean±SE of 5 independent experiments performed in duplicate. **B.** Western blot analysis for CaM, Fas, FLIP, AKT and phospho-AKT. TMX treatment decreased phospho-AKT and FLIP expression but did not affect CaM and Fas levels (n=3).

A.


fold difference (apoptotic cell death)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
TMX

B.

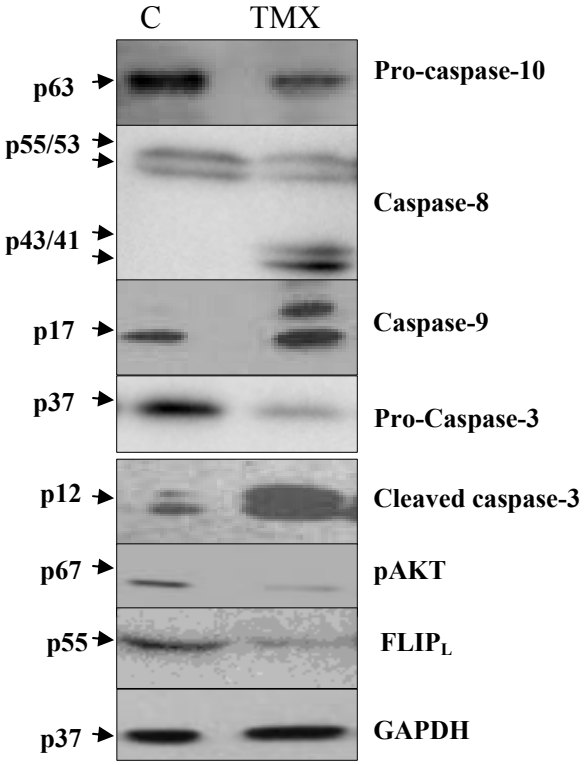
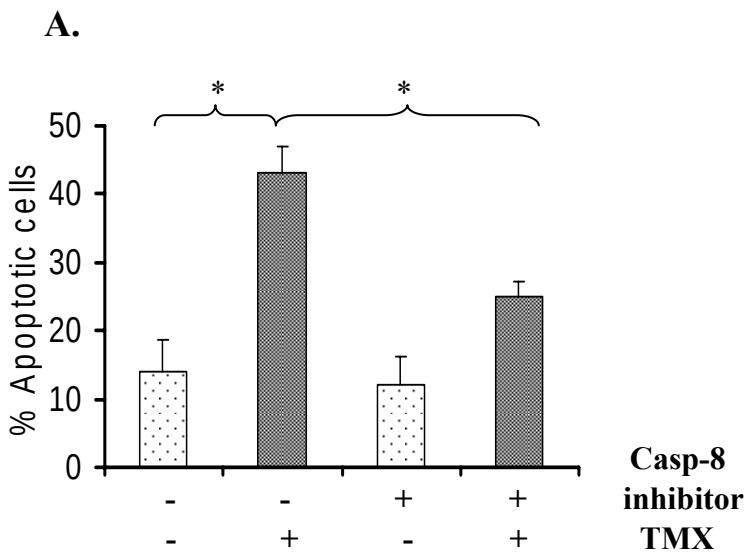
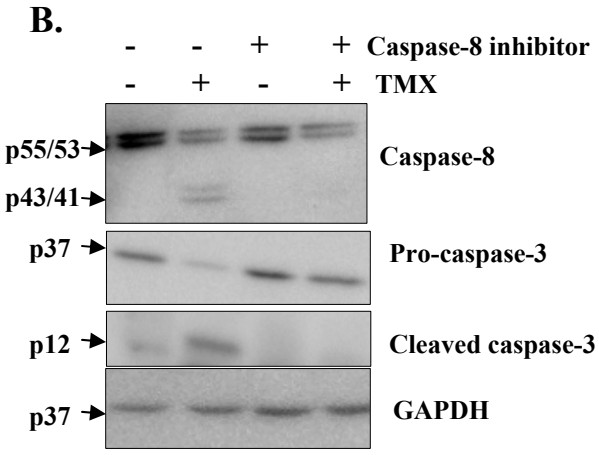

C
TMX
p63
Pro-caspase-10
p55/53
p43/41
Caspase-8
p17
Caspase-9
p37
Pro-Caspase-3
p12
Cleaved caspase-3
p67
pAKT
p55
FLIPL
p37
GAPDH

Figure 3. Analysis of apoptosis markers in response to TMX treatment of cholangiocarcinoma cells

Cholangiocarcinoma cells were treated with 20uM TMX for 24 hrs **A.** Apoptotic cell death was analyzed by AnnexinV/PI staining and expressed as fold difference as compared to control ($n=6$, mean±SE, $p<0.05$). **B.** Western blot analysis showing activation of caspase 8, caspase-10, caspase-3, caspase-9 and inhibition of phospho-AKT and c-FLIP downregulation.


A.
*
*
50
40
30
20
10
0
% Apoptotic cells
- - + + Casp-8 inhibitor
- + - + TMX


B.
- - + + Caspase-8 inhibitor
- + - + TMX
p55/53
p43/41
Caspase-8
p37
Pro-caspase-3
p12
Cleaved caspase-3
p37
GAPDH

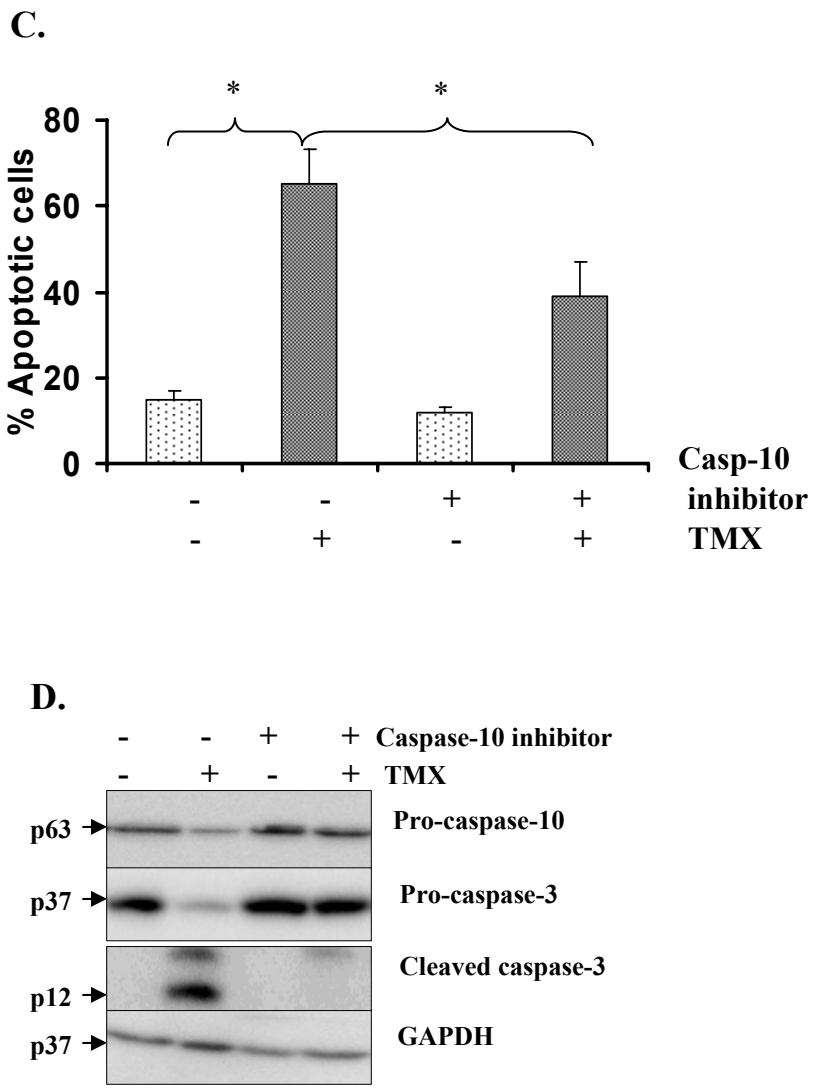
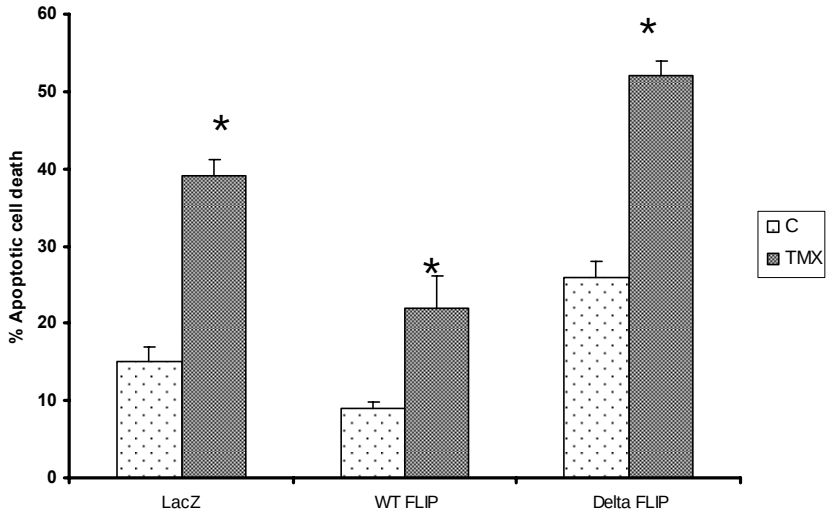


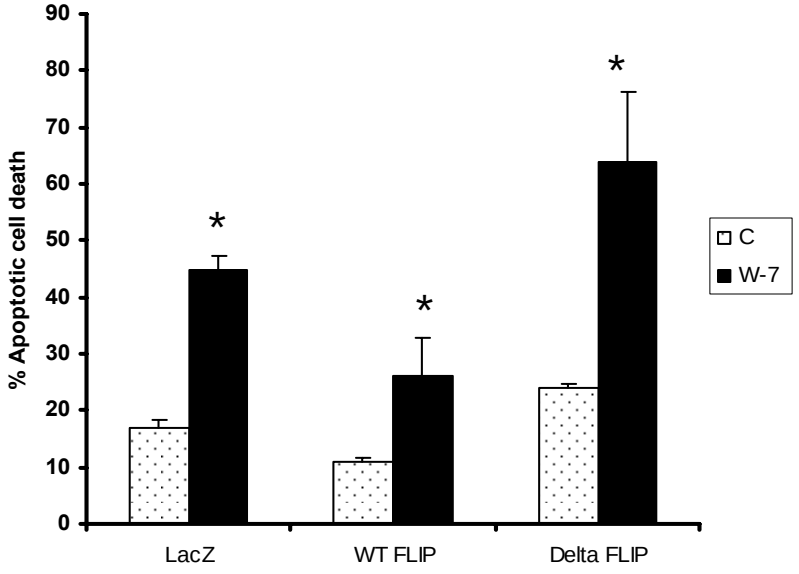
Figure 4. Effect of caspase-8 and caspase-10 inhibitors on TMX induced apoptosis in cholangiocarcinoma cells

Cholangiocarcinoma cells were pretreated with or without **A.** Caspase-8 (50uM) and **C.** Caspase-10 inhibitors (100uM) for 3hours prior to addition of TMX (20uM) for 24 hrs. **B and D.** The whole cell lysates from these cells were analyzed by Western blot for caspase-8, caspase-10, caspase-3, c-FLIP and GAPDH. (n=3, mean±SE, '*' represents $p<0.05$)

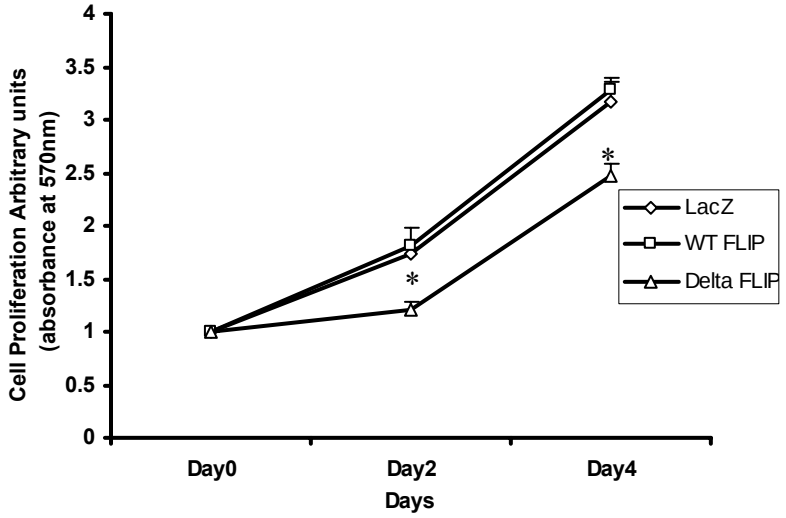
A. TMX


% Apoptotic cell death
60
50
40
30
20
10
0
*
*
*
LacZ
WT FLIP
Delta FLIP
C
TMX

B. W-7


% Apoptotic cell death
90
80
70
60
50
40
30
20
10
0
*
*
*
LacZ
WT FLIP
Delta FLIP
C
W-7

C.


Cell Proliferation Arbitrary units (absorbance at 570nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
Day0
Day2
Day4
Days
*
*
LacZ
WT FLIP
Delta FLIP

D.

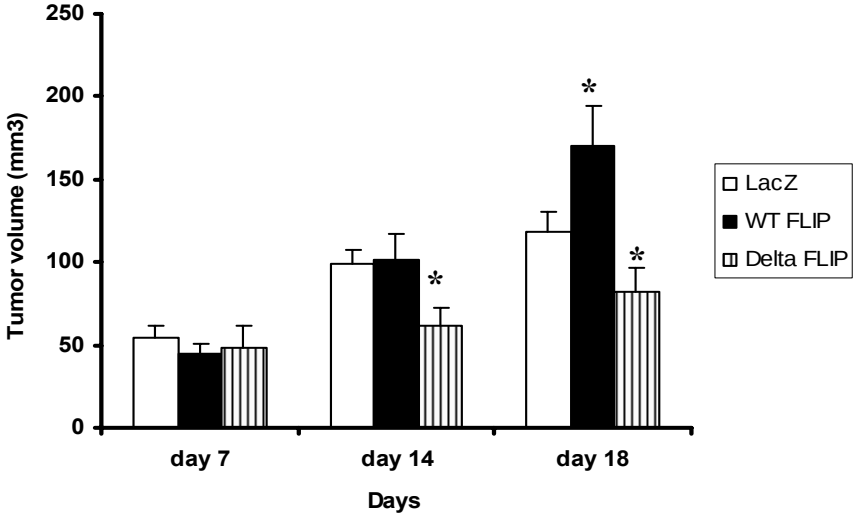

Tumor volume (mm3)
250
200
150
100
50
0
day 7
day 14
day 18
Days
*
*
*
LacZ
WT FLIP
Delta FLIP

Figure 5. Effect of wild type and delta FLIP over expression on TMX induced apoptosis and cell proliferation in cholangiocarcinoma cells

Wild type $FLIP_L$, delta $FLIP_L$ and LacZ proteins were over-expressed in cholangiocarcinoma cells. **A.** The cells were treated with 20uM TMX for 24hrs followed by staining with Annexin V/PI to assess apoptotic cell death (n=3, mean±SE). **B.** The cells were treated with CaM-antagonist W-7 (50uM) for 24hrs followed by assessment of apoptotic cell death (n=4, mean±SE). **C.** The cells were seeded in a 96 well plate and analyzed for cell proliferation using MTT reagent, added on days 0, 2 and 4. The data are representative of at least 3 independent experiments performed in quadruplet. **D.** $2x10^6$ LacZ, WT FLIP and delta FLIP cells were injected in the flanks of 6-7 weeks old athymic nude mice and the tumor growth was followed over 18 days. The tumor volumes were calculated as described in methods section. FLIP expressing cells grew significantly bigger tumors than LacZ and delta-FLIP expressing cells at day 18. Delta-FLIP cells on the other hand produced smallest tumors at day 14 as well as 18. (n=6, mean±SE). '*' represents statistical significance at $p<0.05$ compared with control in each group.

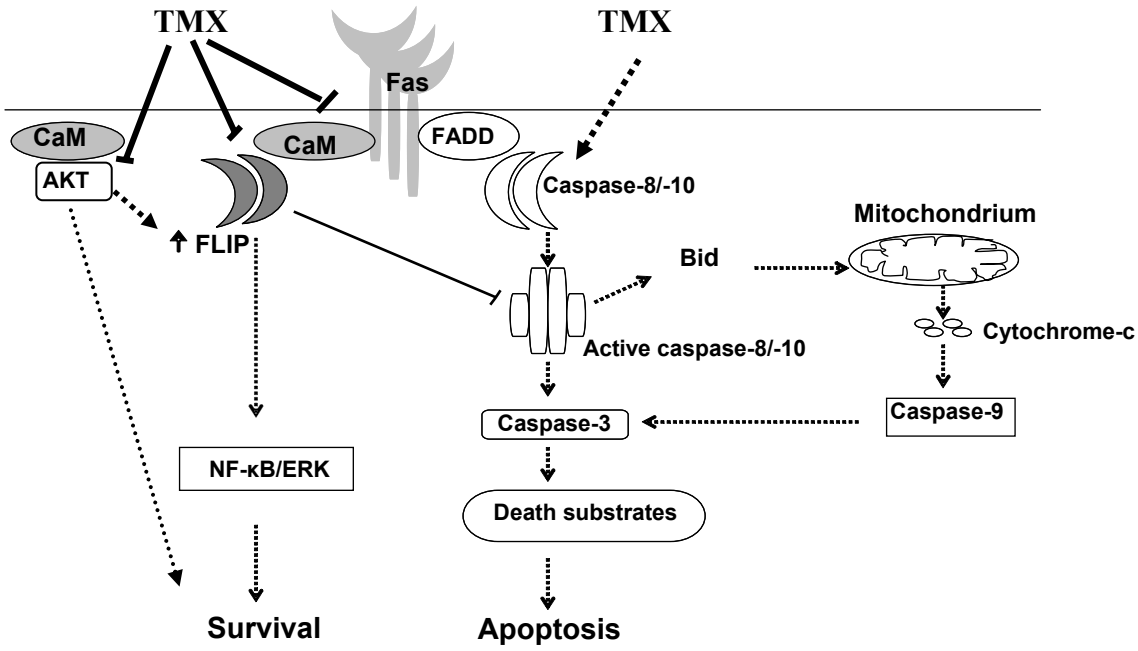


Figure 6. Model for TMX-induced apoptosis
A model depicting the regulation of CaM and Fas signaling by TMX. CaM interacts with Fas, c-FLIP and AKT. TMX interferes with these interactions (shown by bold arrows) to promote apoptotic cell death, similar to Fas-induced apoptosis, and inhibits pro-survival signals at the same time by inhibiting AKT phosphorylation and c-FLIP expression.

----► Indicates activation

—┤ Indicates inhibition

UNPUBLISHED DATA

Effect of the CaM-antagonist, TFP, on FLIP ubiquitination

Our previous work has shown that CaM binding to $FLIP_L$ is regulated during Fas-induced apoptosis and the CaM-antagonist, TFP, interfered with Fas-induced increase in binding between CaM and FLIP [226]. Further, we showed that this TFP mediated decrease in CaM-FLIP binding correlated with decreased phosphorylation of ERK. c-FLIP has been implicated in diverting the Fas-mediated death signals towards survival pathways via activation of pro-survival kinases such as ERK. However, in Fas-sensitive cells which readily undergo apoptosis in response to Fas-activation, FLIP has been reported to undergo proteosomal degradation. This degradation of anti-apoptotic proteins in response to death signals may represent a mechanism adapted by the cells to ensure that cells readily undergo apoptotic cell death by proteolysing inhibitory molecules such as c-FLIP [227]. Thus we investigated the ubiquitination of c-FLIP in response to Fas activation and TFP treatment to test the hypothesis that CaM-FLIP binding is important for stabilization of c-FLIP.

Cholangiocarcinoma cells were treated with Fas activating antibody (500ng/ml) with or without pretreatment with TFP (10uM) for 30 minutes. The cells were lysed and the cell extracts were used for immunoprecipitation using anti-ubiquitin antibody (Affiniti Research Products Ltd. Mamhead, UK). After an overnight incubation at 4ºC, anti-mouse antibody conjugated beads (Sigma, MO, USA) were added to the reaction

followed by incubation for 2 hours. The beads were washed 6 times with lysis buffer followed by western blot analysis using anti-FLIP antibody. Figure 1A shows that treatment of cholangiocarcinoma cells with TFP increased FLIP binding to ubiquitin which paralleled TFP mediated decreased binding between CaM and FLIP in response to Fas activation [226]. Thus the CaM-antagonist, TFP, interfered with Fas-induced increase in CaM-FLIP binding which correlated with decreased phosphorylation of ERK (shown in article 1) and increase FLIP binding to ubiquitin (Fig. 1, unpublished data), suggesting that CaM-FLIP binding may be important for stabilization of c-FLIP.

We analyzed the protein expression levels of CaM and FLIP in these samples after treatment with TFP and Fas. As shown in Fig. 1B, expression of CaM and FLIP was not affected by TFP. Thus in spite of increased FLIP binding to ubiquitin, its protein expression is not affected. One possibility to explain this finding is that these effects were studied over short time points (30 and 60 minutes) and the proteins might not have undergone degradation yet. Further, ubiquitination is a reversible modification of proteins that has been reported to modulate various processes other than proteosomal degradation of proteins, such as endocytosis, gene transcription, DNA repair and replication [228, 229] The process of ubiquitination is characterized by attachment of mono- or ploy-ubiquitin chains to the lysin (K) residues of the substrates. The decision whether to degrade a protein or not depends upon mono- vs poly-ubiquitination and the particular lysine residue that is tagged with ubiquitin chains, Thus attachment of K48 linked poly-ubiquitin chains signal proteosomal degradation of the substrate whereas K63 linked chains, instead of sending the substrate for proteosomal degradation, modulate its ability to participate in various signaling events. Thus the binding of FLIP to ubiquitin may

represent one such modification that only transiently inactivates FLIP, rather than inducing its degradation. Further, it is possible that these effects of TFP on FLIP ubiquitination are due to modulation of some other CaM-dependent pathways by TFP. For example, other mediators such as nitric oxide and TNFά-induced JNK activation have been reported to modulate c-$FLIP_L$ ubiquitination [230, 231]. CaM, through its interaction with nitric oxide synthase (NOS), can regulate the synthesis of nitric oxide (NO) which has been shown to inhibit $FLIP_L$ ubiquitination through its S-nitrosylation.

Nevertheless, it is likely that CaM-FLIP binding may stabilize $FLIP_L$ and increase its availability to interfere with caspase-8 activation as well as mediate the pro-survival signals through Fas pathway.

Activity of Fas-CaM-FLIP axis in the absence of FADD

Fas has been studied extensively as a prototypical death receptor. Recently, many reports identified non-death or pro-survival signaling events mediated through the Fas receptor [92, 232, 233]. Interestingly, initial events after Fas ligation are very comparable in cells that send survival signals instead of death signals via Fas receptor. It has been shown that Fas mediated recruitment of FADD is required as an initial step to recruiting anti-apoptotic proteins such as c-FLIP which can then transduce pro-survival signals [91, 234, 235]. However, there are some reports which suggest that FADD is dispensable for Fas-mediated pro-survival effects, implicating another adapter protein that can substitute for FADD for transmitting survival signals through Fas receptor [233, 236, 237]. Thus after partial hepatectomy in lpr^{cg} mice, which harbor a specific mutation in the Fas cytoplasmic death domain that completely obliterates Fas-FADD binding, Fas

activation using an agonist antibody resulted in rapid regeneration of liver [233]. Wild type mice in which Fas-FADD binding is intact, on the other hand, die within hours of treatment with Fas activating antibody [238]. Similarly, liver-specific expression of dominant negative FADD prevented Fas-induced hepatocyte damage but not NF-kB activation [236, 237]. Therefore there is evidence, both in-vitro and in-vivo, that Fas can transmit survival signals even in the absence of FADD. Thus an alternative pathway is active that operates downstream of Fas even in the absence of FADD. We speculated that CaM, being able to bind both Fas as well as c-FLIP, could provide this alternative pathway in the absence of FADD to transmit survival signals via Fas receptor. Thus we proposed a hypothesis that in the absence of FADD, Fas-CaM-FLIP binding is able to send survival signals in response to Fas activation. In order to test this hypothesis, we used Jurkat T leukemia cells that have been genetically engineered to knock down FADD [239].

Wild type (WT) and FADD deficient (FADD-/-) Jurkat cells were first tested for the expression of FADD and their sensitivity to Fas-induced apoptosis. 1x 10^6 cells were seeded in a 6 well plate and treated with Fas activating antibody (100ng/ml) over 12 hours. The cells were stained with Annexin V and Propidium iodide (PI) to analyze the apoptotic cell death (Annexin V positive and PI negative population was considered to be apoptotic). As shown in Fig. 2, WT cells, which express abundant FADD (western blot shown in insert), are sensitive but FADD-/- Jurkat cells are completely resistant to Fas-induced apoptosis. Next we confirmed the binding of CaM with Fas and c-FLIP in FADD-/- Jurkat cells by using co-immunoprecipitation with anti-CaM antibody as described previously. As shown in Fig.3, Fas and c-FLIP co-precipitated with CaM. CaM

binding to Fas and c-FLIP in WT Jurkat cells has already been established previously [53, 226]. Thus in the absence of FADD, CaM-Fas and CaM-FLIP binding was found to be intact in FADD-/- Jurkat cells.

To investigate if Fas stimulation is able to activate survival signals in the absence of FADD, we treated WT and FADD-/- Jurkat cells with Fas activating antibody (CH-11) and analyzed activation of ERK and NF-kB, two of the important kinases that have been shown to be activated by Fas [89, 92, 232, 233]. Jurkat cells were seeded in a 6 well plate in a serum free medium for 4 hours before addition of the Fas agonist antibody (CH-11, 100ng/ml) for 5, 15 and 30 minutes, followed by immediate cell lysis. The cell lysates were analyzed by western blot for phosphorylation of ERK (p44/42 MAPK) and IkB. As shown in Fig 4, Fas activation did not induce phosphorylation of p44/42 MAPK and IkB in WT Jurkat cells, which is consistent with their ability to undergo Fas-induced apoptosis and not to activate survival signals after Fas activation. In FADD-/- Jurkat cells however, Fas stimulation led to activation of both p44/42 MAPK and IkB, suggesting that the Fas receptor is still able to activate survival signals in the absence of FADD. Since CaM-Fas and CaM-FLIP binding is intact in these cells, it supports the possibility that Fas sends the survival signals through CaM-FLIP binding in FADD-/- Jurkat cells.

CaM interaction with TRAF-2

CaM binds directly with Fas via a classical 1-5-10 CaM binding motif located in the Fas cytoplasmic DD region. The adapter protein, FADD, interacts with Fas via a DD domain as well. Therefore CaM may compete with FADD for Fas binding and thus interfere with DISC aggregation. Further CaM may interfere with aggregation and/or

conformational changes in the Fas receptor that prevent efficient DISC formation. Since CaM-antagonists induce apoptosis and CaM interacts with c-FLIP but not with FADD or caspase-8, it supports the notion that CaM acts as an inhibitor of Fas induced death signaling. We propose that CaM acts as a docking protein for aggregation of pro-survival molecules, such as c-FLIP, to divert death signals towards survival pathway.

c-FLIP is generally considered to be an important molecule in the DISC that can switch the death receptor induced signaling between death and survival pathways. Furthermore, c-FLIP has been reported to interact with TNF receptor associated factor-2 (TRAF-2) and this binding is tought to be essential for FLIP-mediated activation of NFkB. Therefore we determined whether TRAF-2 interacted with CaM as well. We performed binding analysis using protein pull down with CaM-sepharose (CaMS) beads and immunoprecipitation using anti-CaM antibody. Cholangiocarcinoma cell lysates (500ug proteins) were incubated with 60ul 1:1 slurry of CaMS beads overnight at 4ºC in the presence or absence of 2 mM EGTA, a Ca^{++} chelator. In the absence of EGTA, enough Ca^{++} was present in the reaction buffer to bind with CaM. The beads were washed 6 times with lysis buffer and boiled in 2X SDS sample buffer containing 10mM EGTA. The proteins were separated by 12% SDS-PAGE followed by western blot analysis for TRAF-2. Binding between CaM and TRAF-2 was also analyzed by immunoprecipitation using anti-CaM antibody and western blotting for TRAF-2 using a protocol described previously [226]. As shown in Fig. 5, TRAF-2 bound with CaM in Ca++ dependent as well as independent manners. It could be argued that CaM-TRAF-2 binding is mediated via c-FLIP, which has been shown to bind directly with CaM. However CaM-FLIP binding is Ca^{++} dependent, being inhibited by EGTA. Existence of

CaM-TRAF-2 binding even in the presence of EGTA, therefore, makes it unlikely that this binding is mediated via c-FLIP. To our knowledge, a direct binding between c-FLIP and TRAF-2 has not yet been demonstrated. Interestingly, sequence analysis of TRAF-2 for putative CaM binding region using a web based program called the Cellular Calcium Information Server (http://calcium.uhnres.utoronto.ca/), maintained by Dr. Ikura's laboratory at the Ontario Cancer Institute, demonstrated a Ca^{++} independent CaM binding motif, called an IQ motif on its c-terminal region. Therefore we think that there is a strong possibility that CaM-TRAF-2 binding is direct. Furthermore, from our preliminary experiments, other TRAF family members such as TRAF-1, TRAF-5 and TRAF-6 did not bind with CaM (data not shown). Thus CaM may act as a docking protein in death receptor signaling to assemble pro-survival molecules including c-FLIP and TRAF-2 to transmit survival signals. It will be interesting to look for other interacting partners for CaM such as TRADD and RIP. It should however be considered that these effects could be cell type specific as are the abilities of CaM-antagonists to induce apoptosis.

Gemcitabine-induced apoptosis in cholangiocarcinoma cells

Cholangiocarcinoma has remained a very fatal disease with a very poor 5 year survival rate [240, 241]. There are very limited therapeutic options, with conventional chemotherapy and radiotherapy not very effective in the majority of cases and very few patients qualify for surgical resection. Surgical cures do occur but the recurrence rate is very high (about 70%) after surgical resection of the tumor [242]. Thus there is a need for identifying effective therapies. Of the various chemotherapy regimens that are being tried in cholangiocarcinoma patients, 5-fluorouracil (5-FU) has been used extensively as a

single agent or in combination with other drugs with an overall response rate ranging from 0-40% and median survival rate ranging from 2-12 months [243-245]. On the other hand Gemcitabine, a DNA synthesis inhibitor, as a single agent has been shown to have a response rate ranging from 8-60% with a median survival rate ranging from 6-16 months [246, 247]. Therefore we decided to test the efficacy of Gemcitabine in our cholangiocarcinoma tumor model. Initially in order to determine the optimum concentration of Gemcitabine that induced apoptosis in cholangiocarcinoma cells, we treated the cells in vitro with different concentrations for 72 hrs. As shown in Fig. 6A, Gemcitabine induced apoptosis of cholangiocarcinoma cells at 5uM. Further to determine if Gemcitabine-induced apoptosis involved activation of caspases, we probed for the caspases by western blot analysis. As shown in Fig 6B, Gemcitabine predominantly induced activation of caspase-10, -9 and -3.

Several studies have suggested that Gemcitabine alone or in combination with other agents such as cisplatin or oxaplatin represents a well tolerated therapeutic regimen for cholangiocarcinoma [247-249]. Therefore we decided to investigate whether Gemcitabine is effective in our tumor model in combination with the CaM-antagonist, TMX which has been shown to be effective in reducing cholangiocarcinoma growth, in vitro as well as in vivo. TMX is an extensively studied and well tolerated drug that also offers a benefit of a low cost. Therefore we tested the efficacy of combination therapy of Gemcitabine and Tamoxifen in cholangiocarcinoma cells in vitro.

Since 5uM of Gemcitabine was found to be effective in inducing apoptotic cell death, we used this dose for combination therapy with TMX (20uM). Cholangiocarcinoma cells were treated with a combination of TMX and Gemcitabine

over 48hrs and apoptotic cell death was analyzed using Annexin V/Propidium iodide staining. As shown in Fig. 6C, the combination of TMX and Gemcitabine was more effective in inducing apoptotic cell death after 48 hrs treatment than monotherapy with either of the two. Interestingly, both TMX and Gemcitabine seem to involve common caspase pathway that includes activation of caspase-10,-9 and-3.

Caspase-10 requirement for Fas-induced apoptosis in cholangiocarcinoma cells

Here we have shown that both TMX- and Gemcitabine-induced apoptosis involved activation of caspase-10 in cholangiocarcinoma cells. Caspase-10 is an initiator caspase like caspase-8 and it is recruited into the DISC in response to death ligands [34]. To our knowledge, there is no report on the requirement of caspase-10 in Fas-mediated apoptosis in cholangiocarcinoma cells. Therefore, we investigated the effect of caspase-10 and caspase-8 inhibitors on Fas-induced apoptosis in cholangiocarcinoma cells to determine which of these two caspases is essential in the pathway. Cholangiocarcinoma cells, seeded in a 6 well plate, were treated with Caspase-10 (100uM) or caspase-8 (50uM) inhibitors for 3 hours followed by treatment with Fas activating antibody over 24 hours. Apoptotic cell death was analyzed using Annexin V/PI staining. As shown in Fig. 7, both caspase-8 as well as caspase-10 inhibitors completely blocked Fas-induced apoptosis in cholangiocarcinoma cells, suggesting that both caspases are required in this signaling pathway. This is the first report demonstrating that caspase-10, along with caspase-8, is an essential initiator caspase in cholangiocarcinoma cells. The caspase inhibitors however have been reported to loose their specificity by increasing the dose or prolonging the incubation times.

Tumorigenicity of FLIP over expressing cholangiocarcinoma cells in nude mice

In this report, we demonstrated that FLIP is important in mediating resistance, at least partially, to Fas- and TMX-induced apoptosis in our cholangiocarcinoma tumor model. Also in this cholangiocarcinoma tumor model, we demonstrated that the cells resistant to Fas-induced apoptosis expressed significantly increased c-FLIP as compared to the cells which are sensitive to Fas-induced apoptosis. As shown in Fig. 8A, Fas resistant cholangiocarcinoma cells had increased $FLIP_L$ expression as compared to Fas-sensitive cells (n=6, fold difference=1.42±0.12, p=0.01). This is consistent with previous reports implicating FLIP as an inhibitor of Fas mediated apoptosis in several tumor models, including cholangiocarcinoma. In order to determine if FLIP over expression offers any survival benefit to the cholangiocarcinoma cells, we over expressed wild type (WT) FLIP in Fas sensitive cholangiocarcinoma cells and analyzed their sensitivity to Fas-induced apoptosis and tumorigenicity in nude mice. Thus we injected control LacZ or WT FLIP over expressing cells ($5x10^6$ cells in 0.2ul PBS) in the flanks of 6-7 weeks old male athymic nude mice and measured the tumor volumes every 3-4 days over the period of 6 weeks. The tumor volumes were calculated using the formula: volume (mm3) = length x width x width/2. In our previous study reported in chapter 2 in this report, we analyzed the tumorigenicity of FLIP over expressing cells in nude mice over 18 days. In the present studies, however, we allowed the tumors to grow over longer period of time and analyzed the tumor volumes, FLIP expression and apoptotic sensitivity of these cells after 6 weeks. At the end of 6 weeks, mice were sacrificed and tumors were removed under aseptic conditions. The tumors were sliced into smaller pieces and treated with collagenase for 30 minutes at 37ºC. The cells were washed with PBS three times and

grown into culture in complete RPMI 1640 medium. The apoptotic sensitivity of these cells to Fas activating antibody was analyzed by AnnexinV/PI staining and Fas and FLIP expression was analyzed using western blot analysis. As shown in Fig.8B, FLIP over expressing cells grew significantly bigger tumors as compared to control LacZ cells (control=1026±26, WT FLIP=1572±270mm^3, mean±SE, n=10, $p<0.05$). Further the over expression of FLIP was maintained in WT FLIP tumor cells after 6 weeks post injection and the expression of Fas was found to be similar between control and WT FLIP over expressing cells, as shown in Fig. 8C. Consistently, resistance to Fas-induced apoptosis was maintained in the WT FLIP over expressing cultured cells from mice xenografts (% decrease = 40±6%, n=6, $p<0.05$) as shown in Fig. 8D.

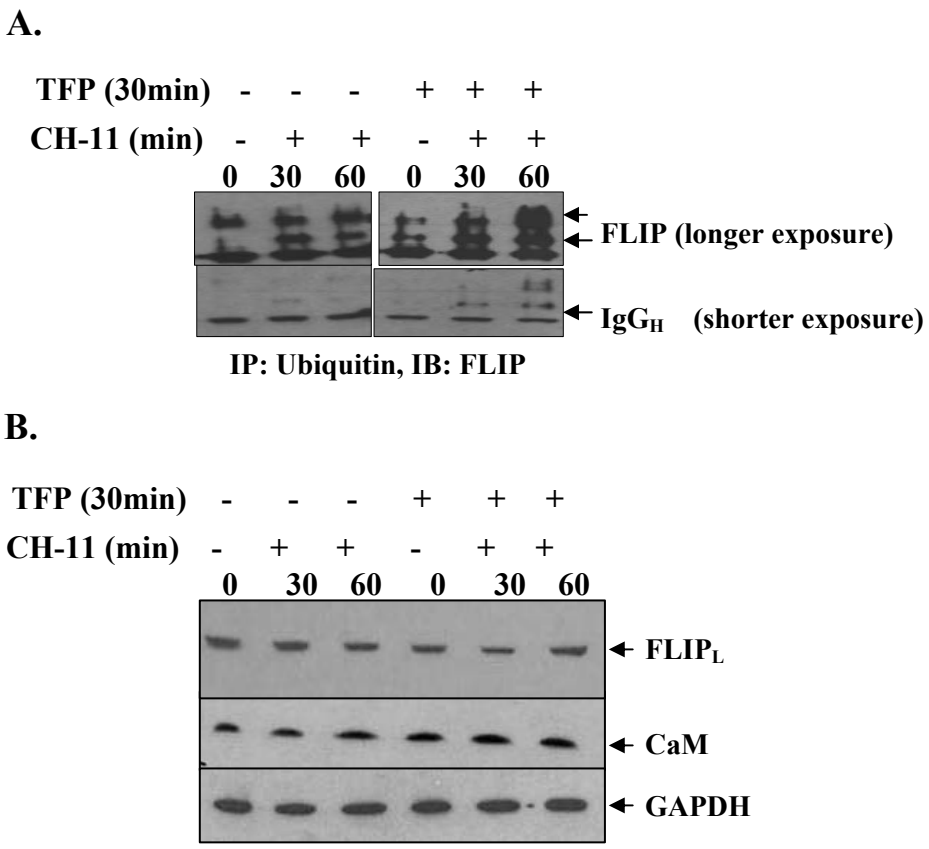


Figure 1. CaM antagonist, TFP, increases $FLIP_L$ binding with ubiquitin
Cholangiocarcinoma cells were treated with Fas agonist antibody (CH-11) with or without pretreatment for 30 minutes with 10uM TFP. **A.** Whole cell lysates (250ug proteins) from the cells were used for co-immunoprecipitation using anti-ubiquitin antibody and immunoblotted for c-FLIP. TFP increased the binding of FLIP with ubiquitin as seen from western blot analysis showing longer (upper panel) as well as shorter (lower panel) exposures. IgG_H indicates heavy chain of the antibody used for IP and shows equal loading of the samples. **B.** Western blot analysis for CaM and FLIP expression in cholangiocarcinoma cells.

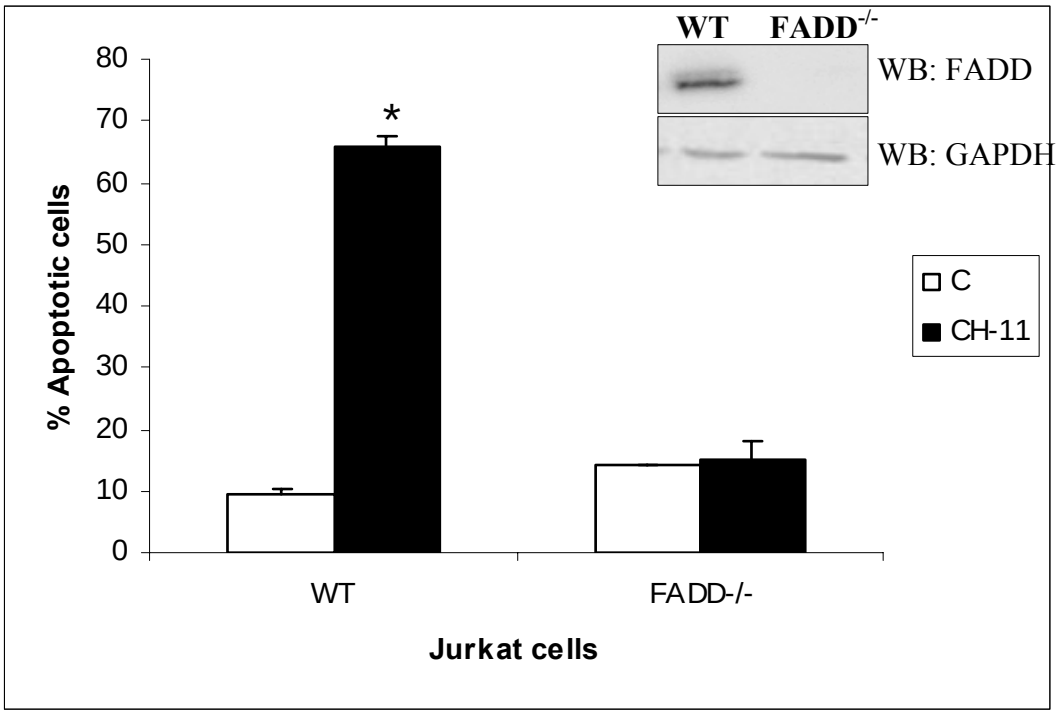


Figure 2. Fas-mediated apoptosis in WT and FADD-/- Jurkat cells
WT and FADD-/- Jurkat cells were treated with Fas agonist antibody (CH-11, 100ng/ml) for 12 hrs. The cells were stained with Annexin V and propidium iodide and analyzed by flow cytometry. The data represent mean±SE (n=3, $p<0.05$). Insert on the right upper corner shows western blot analysis for the expression of FADD in WT and FADD-/- Jurkat cells, showing that it is knocked down completely in FADD-/- Jurkat cells.

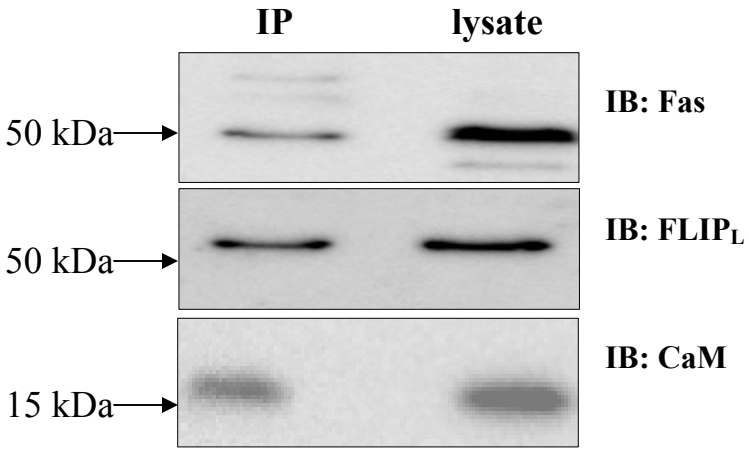


Figure 3. Co-immunoprecipitation of Fas and c-FLIP with CaM in FADD-/- Jurkat cells
FADD-/- Jurkat cell lysates (500ug proteins) were used for immunoprecipitation with anti-CaM antibody. The precipitated proteins were boiled in SDS sample buffer containing 10mM EGTA, separated by 12% SDS-PAGE and western blotted for CaM, Fas and $FLIP_L$. Thus the CaM-Fas and CaM-FLIP binding is intact in FADD-/- Jurkat cells. The data are representative of 3 independent experiments.

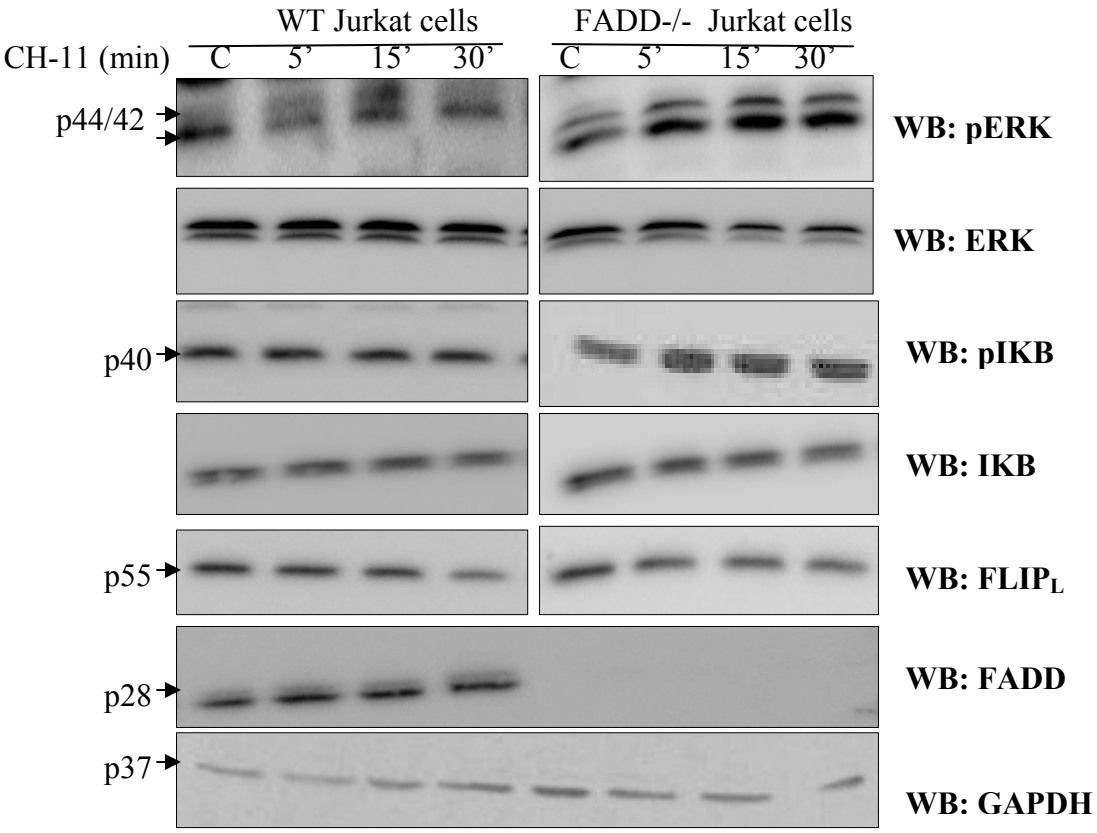


Figure 4. Activation of pro-survival kinases in response to Fas stimulation in FADD-/- Jurkat cells

WT and FADD-/- Jurkat cells were seeded in a 6 well plate a serum free medium for 4 hours. Fas-activating antibody (CH-11) was added for the indicated time course and the cells were immediately lysed after Fas treatment. Cell lysates (50ug proteins) were separated by 12% SDS-PAGE followed by western blot analysis for ERK, IkB, FLIPL and FADD.

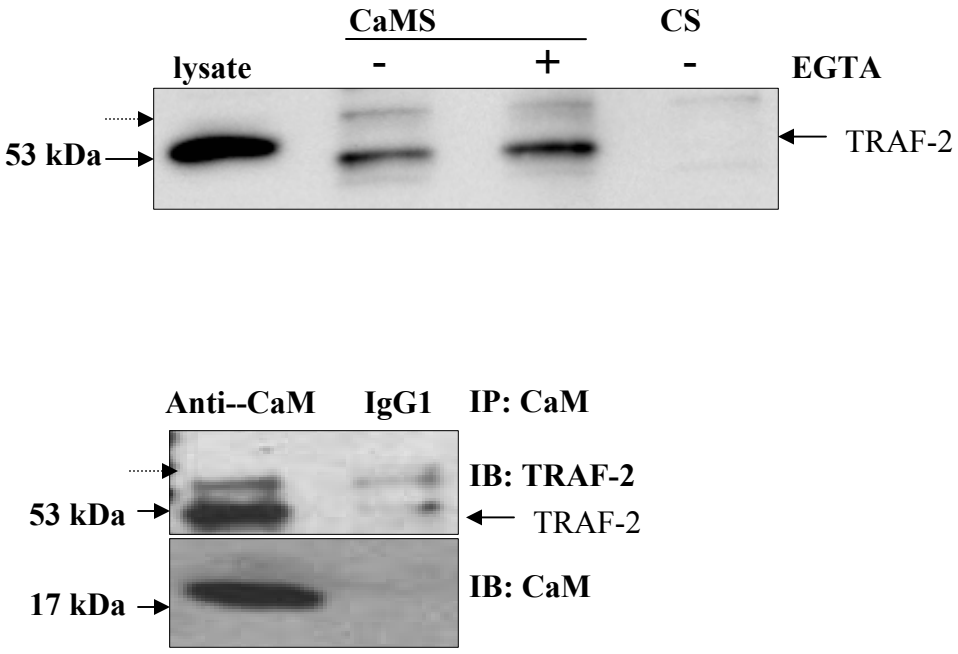
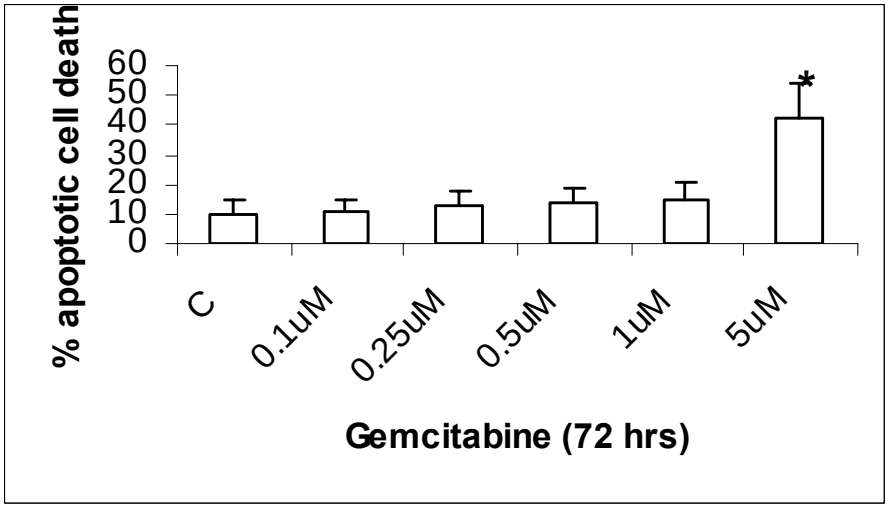
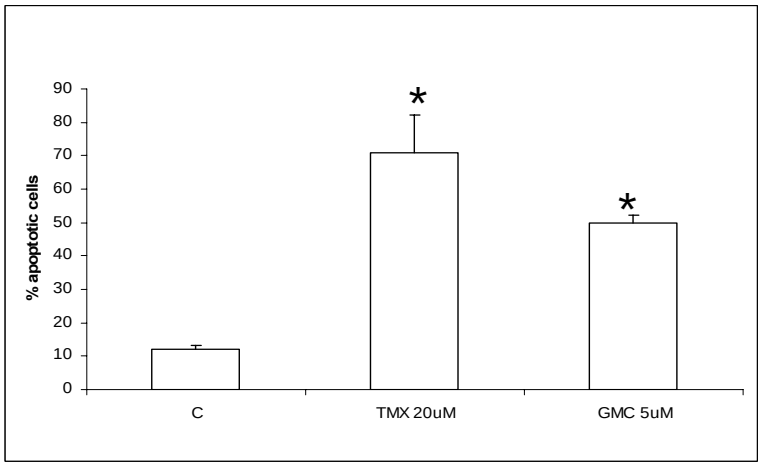


Figure 5. CaM binding with TRAF-2
Cholangiocarcinoma cell lysates were used for protein pull down using CaM-Sepharose (CaMS) or control-sepharose (CS) beads in the presence or absence of 2mM EGTA (upper panel) and anti-CaM or mouse IgG1 antibody for immunoprecipitation (lower panel). TRAF-2 bound specifically with CaMS beads, both in the presence and absence of Ca++ chelator EGTA, but not with CS beads. Also TRAF-2 co-precipitated with CaM in immunoprecipitation using anti-CaM antibody. The upper dotted arrow in both upper and lower panels most likely represent a non specific protein as it can be seen in control lanes in both panels.

A.


% apoptotic cell death
60
50
40
30
20
10
0
*
C
0.1uM
0.25uM
0.5uM
1uM
5uM
Gemcitabine (72 hrs)

B.


% apoptotic cells
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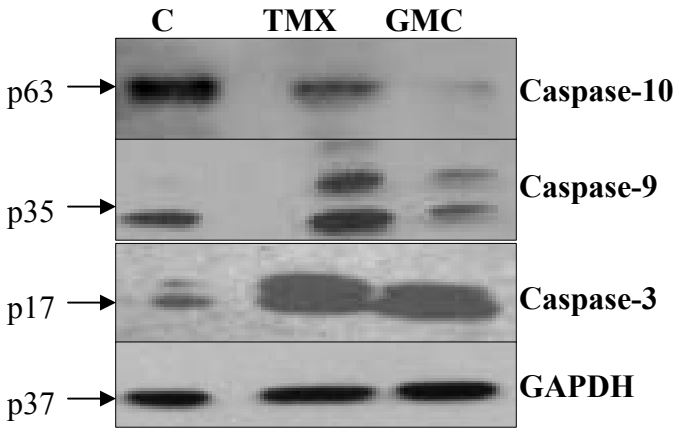
80
70
60
50
40
30
20
10
0
*
*
C
TMX 20uM
GMC 5uM


C
TMX
GMC
p63
Caspase-10
p35
Caspase-9
p17
Caspase-3
p37
GAPDH

C.

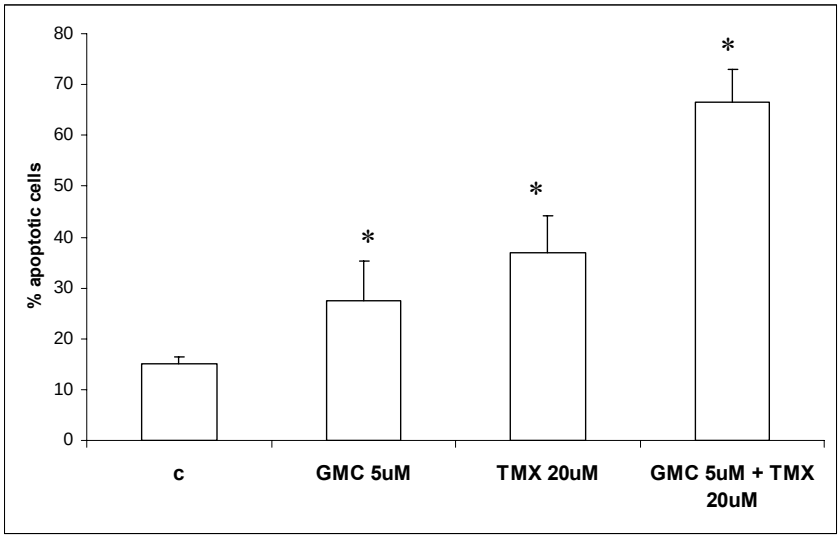
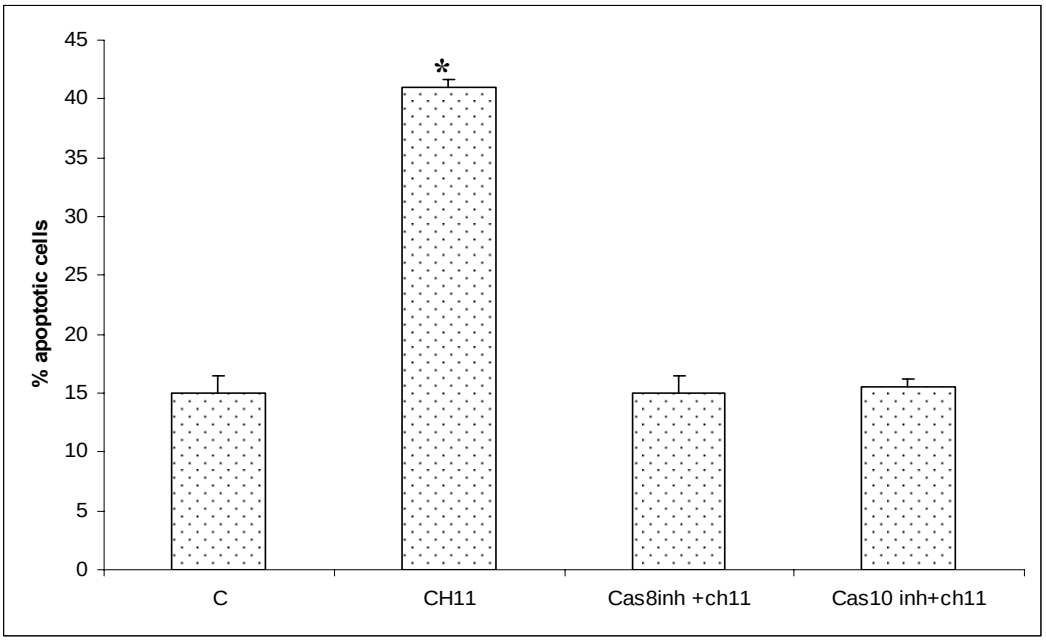


Figure 6. Gemcitabine induced apoptosis in cholangiocarcinoma cells
A. Cholangiocarcinoma cells were treated with various doses of Gemcitabine (GMC) as shown in the figure for 72 hours. The apoptotic cell death was determined by staining the cells with AnnexinV/Propidium iodide (PI) and counting AnnexinV positive and PI negative cells. The data represent mean±SE (n=2, $p<0.05$). **B.** Cholangiocarcinoma cells were treated with TMX (20uM) and GMC (5uM) over 72 hours and apoptotic cell death was analyzed by Annexin V/PI staining as shown in upper panel in Fig. 6B. The data represent mean±SE (n=3, $p<0.05$). Lower panel in Fig 6B shows western blot analysis of cell lysates from TMX and GMC treated cells, showing activation of caspase-10 (seen as increased processing of precursor form p67), -9 and-3 (seen as increased formation of cleaved products, p17 and p12 respectively) in response to these agents, indicating that the cell death induced by TMX and GMC involves activation of the caspase cascade. GAPDH is used as a loading control. A representative result from three independent experiments is shown. **C.** Cholangiocarcinoma cells were treated with TMC, GMC or TMX and GMC combination for 48 hours and apoptotic cell death was analyzed, showing that combination therapy was more effective in vitro than monotherapy with either drug. The data represented as mean±SE (n=3, $p<0.05$).

A.


45
40
35
30
25
20
15
10
5
0
% apoptotic cells
*
C
CH11
Cas8inh +ch11
Cas10 inh+ch11

B.

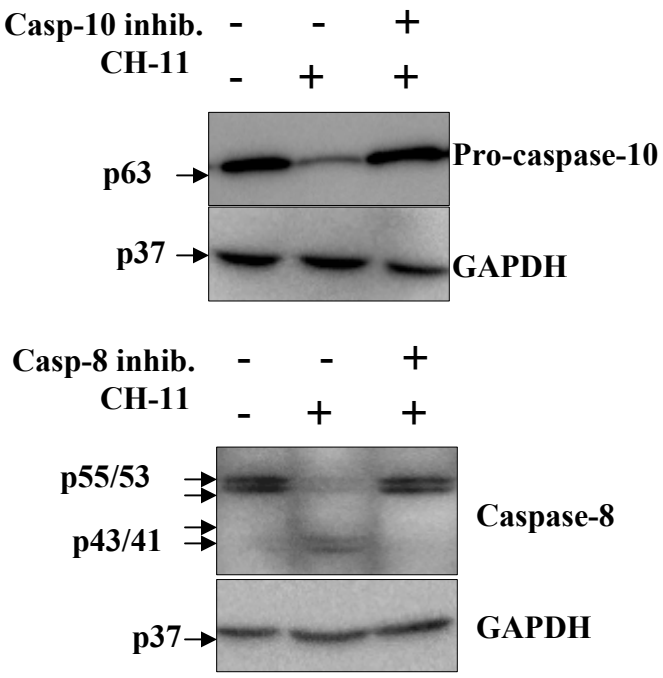
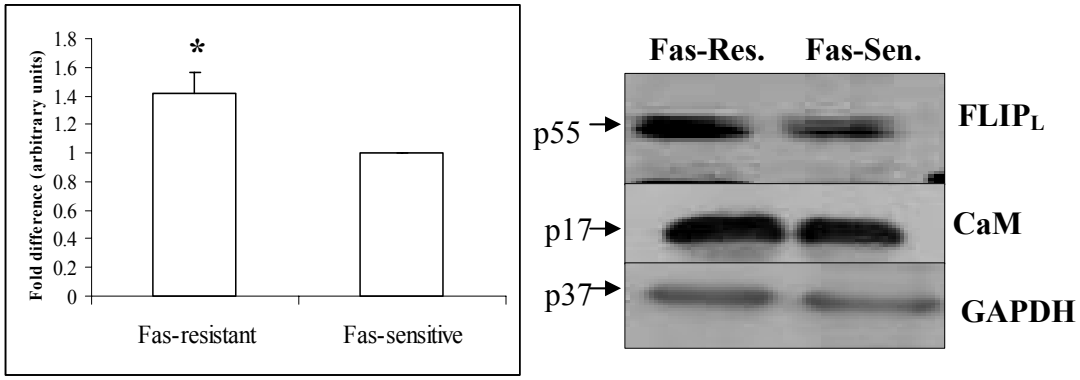

Casp-10 inhib. - - +
CH-11 - + +
p63
Pro-caspase-10
p37
GAPDH
Casp-8 inhib. - - +
CH-11 - + +
p55/53
p43/41
Caspase-8
p37
GAPDH

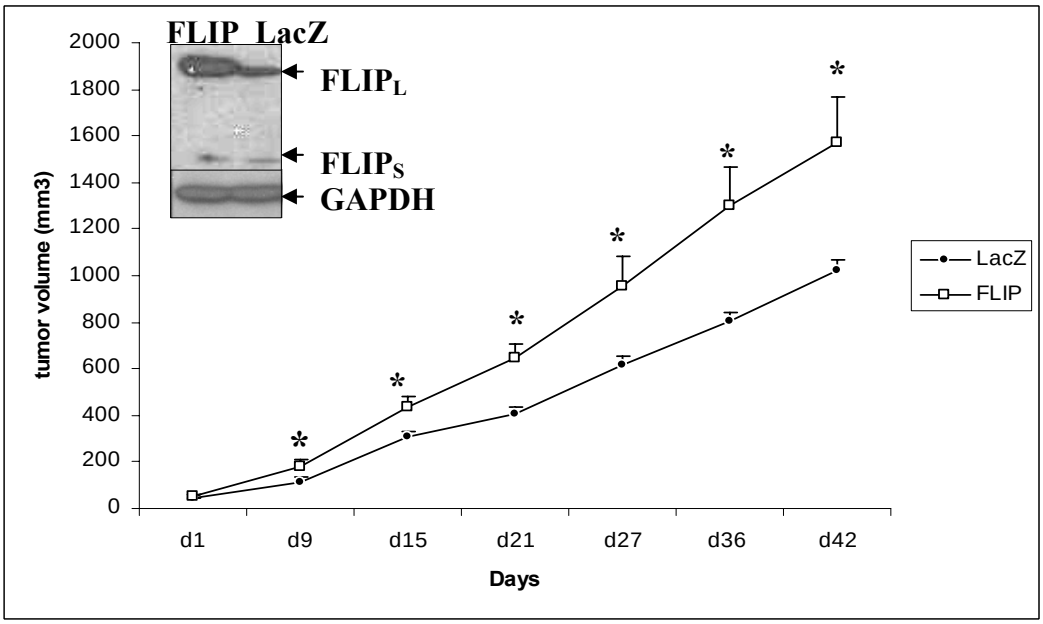
Figure 7. Requirement of caspase-10 in Fas-induced apoptosis in cholangiocarcinoma cells

A. Cholangiocarcinoma cells were pretreated with inhibitors of caspase-8 (50uM) and -10 (100uM) for 3 hrs followed by incubation with Fas activating antibody for 24 hrs. Apoptotic cell death was analyzed using flow cytometry after staining the cells with AnnexinV and PI. Inhibition of either caspase-8 or-10 results in complete blockade of Fas-induced apoptosis suggesting that both caspases are required to initiate the apoptotic cascade. The data represent mean±SE ($n=2$, $p<0.05$). **B.** Western blot analysis of the cell lysates treated with caspase inhibitors for caspase-8 and -10 shows that both caspase inhibitors effectively blocked the activation of respective caspases. Fas activating antibody induces activation of both initiator caspases; caspase-8 (seen as increase in processed p43/41 forms and decrease in precursor p55/53 forms) and caspase-10 (seen as decrease in precursor p63 form).

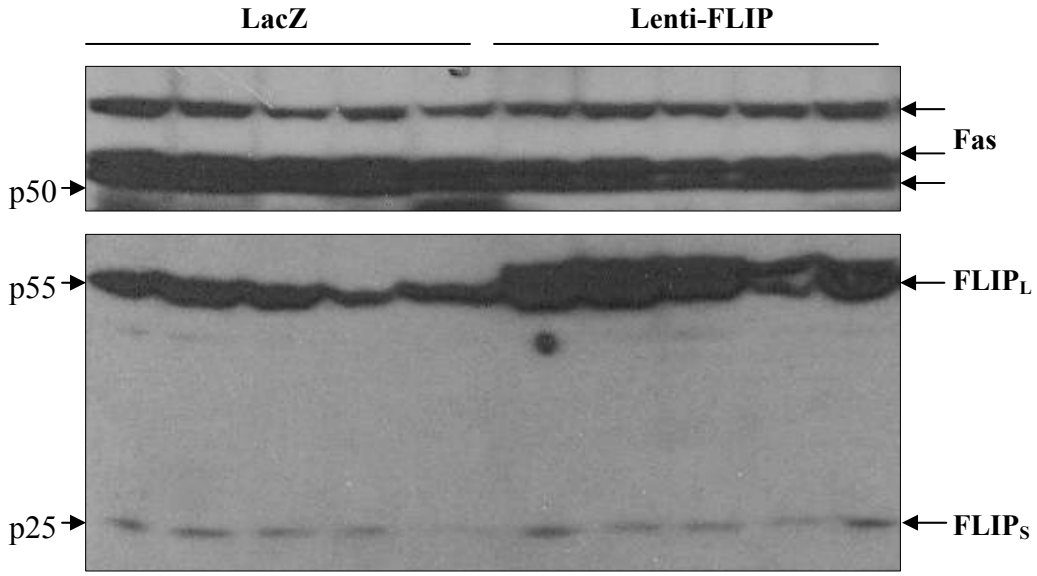
A.


Fold difference (arbitrary units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Fas-resistant
Fas-sensitive
Fas-Res.
Fas-Sen.
p55
p17
p37
FLIPL
CaM
GAPDH

B.


FLIP LacZ
FLIPL
FLIPS
GAPDH
tumor volume (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
d1
d9
d15
d21
d27
d36
d42
Days
LacZ
FLIP

C.


LacZ
Lenti-FLIP
Fas
p50
p55
FLIP$_L$
p25
FLIP$_S$

D.

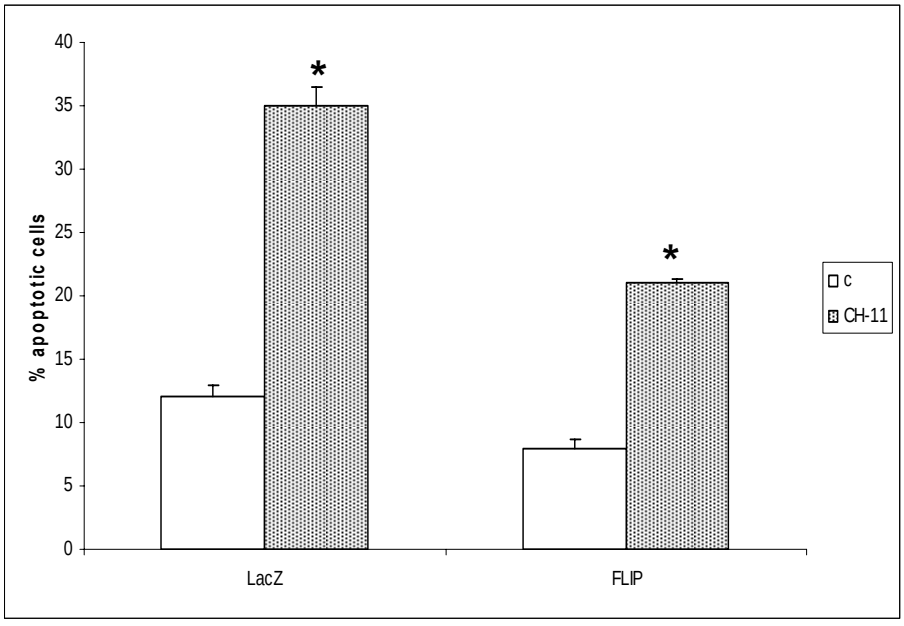

% apoptotic cells
40
35
30
25
20
15
10
5
0
*
*
LacZ
FLIP
c
CH-11

Figure 8. Tumorigenesis of wild type FLIP over expressing cholangiocarcinoma cells in nude mice

A. Cell lysates from Fas-sensitive and Fas-resistant cholangiocarcinoma cells were analyzed for FLIP expression by western blot. $FLIP_L$ expression was quantitated by measuring the band intensities of FLIP using densitometry and normalizing these with GAPDH band intensities using Adobe photoshop application. As shown in the figure, FLIP expression was increased significantly in Fas-resistant cells as compared to Fas-sensitive cells. The data represented as mean±SE ($n=6$, $p<0.05$). **B.** Cholangiocarcinoma cells stably over expressing wild type (WT) FLIP or LacZ as a control were injected bilaterally into the flanks of 6-7 weeks old athymic male nude mice. The tumor volumes were measured every 3-4 days over the period of 6 weeks. The FLIP over expressing cells grew significantly bigger tumors, starting from day 9, as compared to control cells. The data are represented as mean±SE ($n=10$, $p<0.05$). The insert on upper left panel shows the over expression of $FLIP_L$ achieved with lentiviral vector in cholangiocarcinoma cells as compared to LacZ control. Note that the expression of $FLIP_S$ is not altered between these two cell lines. GAPDH is used as a loading control. **C.** At the end of the experiment at day 42, the mice were sacrificed and the tumor cells from mice xenografts were grown into culture to analyze the expression of Fas and FLIP. As shown in the figure, Fas expression was found to be identical among 5 tumor samples tested from each group. Further, FLIP over expression was maintained over the period of 6 weeks post tumor cell injection. **D.** The apoptotic sensitivity of these cells to Fas induced apoptosis was determined using Fas activating antibody treatment over 24 hours. Consistent with pre-injection response, the FLIP over expressing cells were significantly more resistant to Fas-induced apoptosis as compared to LacZ cells. The data represented as mean±SE ($n=6$, $p<0.05$).

SUMMARY OF UNPUBLISHED DATA

The experiments presented in this section were aimed at further characterizing the biology of CaM-FLIP interaction with respect to Fas signaling and to explore the possible avenues for therapeutic interventions in cholangiocarcinoma. We demonstrated:

- CaM-FLIP binding is important in stabilization of c-FLIP. Inhibition of the Fas-induced increase in CaM-FLIP binding by the CaM-antagonist, TFP, is correlated with increased binding of c-FLIP to ubiquitin and concurrent inhibition of ERK phosphorylation. These data suggest that CaM-FLIP binding may be important for maintaining FLIP mediated survival signals.
- FADD deficient Jurkat cells were used to further test the hypothesis that CaM-FLIP binding is important in sending survival signals after Fas activation. In the absence of the key adapter protein FADD, CaM-Fas and CaM-FLIP binding were found to be intact in these cells. Further the Fas activation was able to activate ERK and NF-kB pathways in FADD deficient but not in wild type Jurkat cells, suggesting that Fas-CaM-FLIP signaling axis was responsive to Fas activation in the absence of FADD in Jurkat cells.
- Another important pro-survival protein, TRAF-2, was shown to interact with CaM in Ca^{++} dependent and independent manners, further supporting the notion that CaM may act as a docking protein for the aggregation of various pro-survival

proteins such as c-FLIP and TRAF-2 to send survival signals downstream of death receptors.

- FLIP over expression increased the tumorigenicity of cholangiocarcinoma cells in nude mice.
- Both caspase-8 and caspase-10 were shown to be essential in Fas and TMX-induced apoptosis in cholangiocarcinoma cells.
- Gemcitabine, a commonly used drug in cholangiocarcinoma therapy, was found to be effective in inducing apoptosis in human cholangiocarcinoma cells.
- The CaM-antagonist, TMX, when used in combination with Gemcitabine in cholangiocarcinoma cells showed enhanced induction of apoptosis compared to monotherapy with either drug, suggesting that the combination might be beneficial for treating patients.

These data support the concept that CaM-FLIP binding plays an important role in modulation of the Fas signaling and that CaM-antagonists, such as TMX, either alone or in combination with Gemcitabine, might potentially be used for therapy in cholangiocarcinoma.

CONCLUSION

The ultimate goal of the research presented in this thesis is to identify novel chemotherapeutic agents for treating lethal tumors like cholangiocarcinoma. The incidence of cholangiocarcinoma in the Unites States has been increasing over the past few years [189, 190]. Because of difficulties with timely diagnosis and ineffective therapies, the overall 5 year survival rate has remained dismal: less than 9% following diagnosis. Hence there is an urgent need to identify novel strategies for therapeutic intervention in this fatal disease. Based on the prior work from our group implicating Ca^{++}-CaM and Fas in cholangiocarcinoma tumorigenesis, we speculated that understanding the molecular mechanisms involved in the modulation of Fas signaling by CaM could identify molecular targets that can be used in early diagnosis and/or therapy for cholangiocarcinoma. More specifically this work sought to characterize the cross talk between Fas and CaM signaling pathways and identify novel therapeutic targets.

Fas is a well characterized death receptor that is expressed in various cell types in the body and has been studied extensively with respect to its apoptosis inducing properties. As an example, the Fas mediated death pathway has been shown to be critical in lymphocyte homeostasis and is used to eliminate activated or auto reactive lymphocytes from the body [250]. In cholangiocarcinoma tumors from patients, Fas expression has been shown to correlate negatively with disease progression and patient survival such that expression of the Fas receptor is decreased with progressive disease

[198, 199]. Thus it might be used as a molecular marker and potentially as a therapeutic target in cholangiocarcinoma.

Ligation of the Fas receptor with FasL results in recruitment of an adapter protein, FADD, which then recruits proteins such as caspase-8, -10 and/or c-FLIP (Fig 1, highlighted in grey) to form the death inducing signaling complex (DISC). The initiator caspases undergo cleavage in the DISC, triggering the cascade of caspase activation to induce apoptotic cell death. In addition to its established role as a death receptor, recent work by many investigators has underscored the importance of the Fas pathway in non-apoptotic signaling [49, 92, 232, 233]. Further, it has been shown that some of the molecular machinery used to send survival signals is similar to that used in the death inducing pathway. For example increased recruitment of FLIP into the DISC prevents activation of initiator caspases and inhibits down stream apoptotic signaling. Additionally, FLIP recruitment into the DISC has also been associated with activation of survival pathways such as NF-κB and ERK. However the molecular determinants that allow FLIP to switch the signaling from death towards survival are not well understood.

Unlike Fas, the TNF receptor mediated survival signaling is well characterized. After binding with ligand, TNF-ά, the formation of a specific TNF receptor associated complex of proteins determines which signal is transmitted downstream [251]. Thus when the adapter protein TRADD (TNF receptor associated death domain containing protein) recruits the FADD-caspase complex, apoptotic signaling is initiated. But when TRADD recruits TRAF-2 or RIP (receptor interacting protein) it activates pro-survival signaling [252, 253]. Therefore, it appears that the aggregation of specific proteins

downstream of the receptor is critical in switching the signaling from death towards survival pathways.

In this context, previous studies by our group contributed a novel perspective to Fas pathway by demonstrating cross talk between Fas and Ca^{++}-CaM signaling. Ahn et al showed that CaM-antagonists induced apoptosis in cholangiocarcinoma cells using mechanisms similar to those used in Fas-induced apoptosis [55, 56, 188, 200]. Further, they demonstrated that CaM interacts with the Fas receptor via a classical, Ca^{++} dependent, 1-5-10 CaM binding motif located in the death domain (DD) of Fas cytoplasmic tail [53]. Fas was the only member of the TNF superfamily of receptors interacting with CaM, further underscoring the putative role of CaM in Fas mediated signaling. Furthermore, we demonstrated that CaM is recruited into the Fas mediated death inducing signaling complex (DISC) after Fas activation (shown in Fig.1) suggesting that the dynamics of Fas-CaM binding might be important in modulating the formation of the DISC and downstream signaling [54]. To characterize the role of CaM in Fas signaling, we screened for other interacting partners for CaM in the Fas-induced DISC. We identified that only $FLIP_L$, an anti-apoptotic protein that interferes with Fas-induced apoptosis, interacted with CaM, and other DISC proteins including, FADD, caspase-8 and $FLIP_S$ did not interact with CaM (Fig. 1 in article 1) [226]. Characterization of CaM-FLIP binding revealed that it is a Ca^{++} dependent, direct interaction that is regulated in response to Fas- and CaM-antagonists induced apoptosis and by changes in Ca^{++} levels (Fig. 2, 3 and 4 in article 1). Further, the CaM binding region was localized to amino acids 197-213 on C-terminal region of $FLIP_L$ (Fig. 8 in

article 1). Interestingly, unlike Fas which binds CaM via a classic 1-5-10 motif, the CaM binding region on c-FLIP appears to have a unique sequence.

The Ca^{++} dependent direct binding of CaM with Fas and $FLIP_L$ is a novel phenomenon in Fas signaling that might address some of the ambiguity in the Fas-induced non-apoptotic signals. Considering the ability of CaM-antagonists to induce apoptosis in cholangiocarcinoma cells and the direct interaction of CaM with Fas and an anti-apoptotic protein, c-FLIP, we speculate that CaM is important in mediating pro-survival signals in Fas pathway. Importantly, the CaM binding region on the Fas DD is located in the same helix that is utilized in Fas-FADD binding. Hence, there is a possibility that in response to Fas stimulation, both FADD and CaM compete for binding with the Fas DD. After Fas activation, there is a Ca^{++} dependent increase in CaM-FLIP binding and increased recruitment of CaM into the DISC as well. Therefore another speculation supporting the inhibitory role of CaM in Fas apoptotic pathway is that it provides an additional pathway for recruitment of $FLIP_L$ into the Fas mediated DISC. Since relative amounts of caspases and FLIP are critical in deciding whether death or survival signals are transmitted through the DISC, this additional pathway of $FLIP_L$ recruitment via CaM might be critical in situations where FLIP expression is increased relative to caspases. Interestingly, $FLIP_L$ has been suggested to be a more potent inhibitor of apoptosis than $FLIP_S$. It is possible that the dynamics of the binding of $FLIP_L$ with CaM renders it more effective than $FLIP_S$ in inhibiting apoptotic signaling.

As a part of the functional characterization of CaM-$FLIP_L$ binding, we demonstrated that this binding is dynamic, increasing significantly in response to Fas activation (Fig. 3 in article 1). Inhibition of CaM-FLIP binding using the CaM-

antagonist, TFP, concurrently inhibited ERK activation (Fig. 5 in article 1) and increased the binding of $FLIP_L$ with ubiquitin (Fig. 1 in unpublished data). Thus we speculated that CaM-FLIP binding is important for stabilization of c-FLIP. Stable over expression of wild type (WT) $FLIP_L$, with intact binding to CaM, rendered cholangiocarcinoma cells significantly resistant to both Fas as well as CaM-antagonist-induced apoptosis and increased their tumorigenicity in nude mice. Interference with CaM-FLIP binding by over expressing mutant $FLIP_L$ protein with deletion of CaM binding region (Delta-FLIP) restored the sensitivity of cholangiocarcinoma cells to both Fas and CaM-antagonist-induced apoptosis (Fig. 9 in article 1 and Fig. 5 in article 2). Thus it appears that CaM binding to FLIP is important in mediating the apoptosis inhibitory effects of c-FLIP. In addition, deletion of CaM binding region from FLIP increased spontaneous apoptosis and decreased the tumorigenicity of cholangiocarcinoma cells in nude mice xenografts. Thus CaM-$FLIP_L$ binding modulates the anti-apoptotic/pro-survival effects of c-FLIP and is important in cholangiocarcinoma tumorigenesis. It is possible that disruption of CaM binding region causes conformational changes in $FLIP_L$ affecting its recruitment into the DISC and/or activation. Another possibility is that the mutant $FLIP_L$ acts as a dominant negative protein, interfering with the functions of endogenous FLIP. Further characterization of the molecular events leading to the restoration of the apoptotic sensitivity of cholangiocarcinoma cells with delta FLIP protein expression is necessary to fully understand the functional significance of CaM-FLIP binding. Investigations are warranted to analyze the interaction of delta FLIP protein with other interacting partners such as FADD, TRAF-2 and RIP and their recruitment into the DISC. With our preliminary studies indicating an interaction between CaM and TRAF-2 (Fig 5,

unpublished data), it is likely that CaM acts as a docking protein to form a signaling complex that transmits pro-survival signals through Fas under appropriate conditions. It will be interesting to study the interaction of CaM with FLIP and possibly TRAF-2 with respect to other death receptors such as TRAIL and TNF-R1. Understanding CaM-FLIP biology in the context of death receptor signaling may help us elucidate the molecular mechanisms by which CaM-FLIP is able to act as a switch that diverts death signaling towards survival in response to the same ligand or even in ligand independent manners.

Protein-protein interactions are important in regulation of apoptotic signaling. The structural changes and the dynamics of the protein interactions such as CaM-Fas, CaM-FLIP and Fas-FADD bindings, during the formation of Fas-induced signaling complex can be analyzed using both computational modeling and structural biology approaches. Such studies will provide important information about the conformational changes, sequence of events/interactions, competitions, and possibly the important sites on CaM involved in interactions with Fas and c-FLIP. The findings may enable us to develop specific compounds to target desired interactions which can be used potentially in cancer therapy and in other diseases where alterations in apoptosis are implicated in disease pathophysiology.

From a therapeutic point of view, we investigated the use of TMX, a potent CaM-antagonist, in vitro and in vivo in a nude mouse model in this dissertation. TMX, a well known and well studied estrogen receptor modulator, has been proven to be safe even at high doses. For example serum levels ~3uM of TMX and its metabolite 4-hydroxytamoxifen were reported in patients receiving TMX [254]. We investigated its efficacy in reducing the tumor size of cholangiocarcinoma xenografts in a nude mouse

model and showed that both intra-peritoneal as well as intra-tumoral injections of TMX were effective in inhibiting tumor growth. Thus a dose of TMX as small as 3mg/kg (0.1mg per mouse) was found to be effective in reducing tumor size in nude mice in our studies. In order to reproduce the serum levels of TMX similar to those observed in breast cancer patients, a dose of 200mg/kg of TMX orally or at least 25mg/kg intra-peritoneally is needed in mice [255, 256]. By comparison the doses that were used in our studies were very small (3mg/kg). Further studies using TMX doses equivalent to those in human patients are needed to confirm its effect on cholangiocarcinoma tumorigenesis. We speculate that with increased doses of TMX, a better reduction in the size of the cholangiocarcinoma xenografts in the nude mice model is possible and will hopefully help us translate these findings for use in patients.

As a part of the molecular characterization of TMX-induced apoptosis, we showed that it involves caspase-dependent as well as independent pathways. It induced activation of caspase-8, -10, -9 and -3, indicating the involvement of the caspase cascade in the apoptotic death (Fig. 3 in article 2). Further TMX inhibited the expression of c-FLIP and activation of AKT, both CaM binding pro-survival proteins (Fig. 3 in article 2). Inhibition of pAKT has been shown to correlate with decreased FLIP expression in many tumors. Thus it is possible that the effect of TMX on FLIP expression is mediated via pAKT. We also demonstrated that another CaM-antagonist, TFP, increased FLIP binding with ubiquitin (Fig.1, unpublished data). Thus there is a possibility that TMX, like TFP, enhances FLIP binding with ubiquitin and thus decreases FLIP protein expression via increased proteosomal degradation of FLIP. Further investigation to determine whether FLIP expression is affected at the mRNA level or the protein level is warranted. From

these observations, it appears that TMX induces apoptosis of cholangiocarcinoma cells by activation of caspases and inhibiting pro-survival signals simultaneously by down regulating pAKT and FLIP, probably by affecting CaM-AKT and CaM-FLIP interactions as shown in Fig. 1. Therefore, there is a strong evidence that TMX induced apoptosis in cholangiocarcinoma involves modulation of CaM signaling.

Of the several chemotherapeutic agents that are currently being used in the management of cholangiocarcinoma, Gemcitabine has been shown to have a favorable efficacy and toxicity profile [246, 247]. Therefore we investigated the apoptosis inducing abilities of Gemcitabine in our cholangiocarcinoma cell lines. We showed that Gemcitabine induced apoptosis of cholangiocarcinoma cells in a dose dependent manner, probably by utilizing a cascade of caspase activation like TMX (Fig. 6, unpublished data). Whether it uses caspase independent pathways, like TMX, is still to be determined. Analyzing the efficacy of combination therapy of Gemcitabine and TMX in vitro showed that the combination was more effective than monotherapy with either drug (Fig. 6, unpublished data). This needs to be investigated further, particularly in cholangiocarcinoma xenograft model in nude mice.

From the studies presented in this report it is clear that CaM plays an important role in modulation of Fas signaling in cholangiocarcinoma via its direct interaction with c-$FLIP_L$. Considering the multitude of signaling pathways modulated by CaM and the hierarchical placement of c-FLIP in apoptotic signaling, CaM-FLIP binding may provide a novel and effective therapeutic target for cholangiocarcinoma and possibly other lethal tumors in which Fas and c-FLIP expression are implicated in the disease pathophysiology. Furthermore, CaM-antagonists, like TMX, can be used potentially as a

therapy in cholangiocarcinoma. The combination therapy with drugs like TMX, Gemcitabine or compounds disrupting CaM-FLIP and/or CaM-Fas binding specifically may provide promising therapeutic approaches in cholangiocarcinoma.

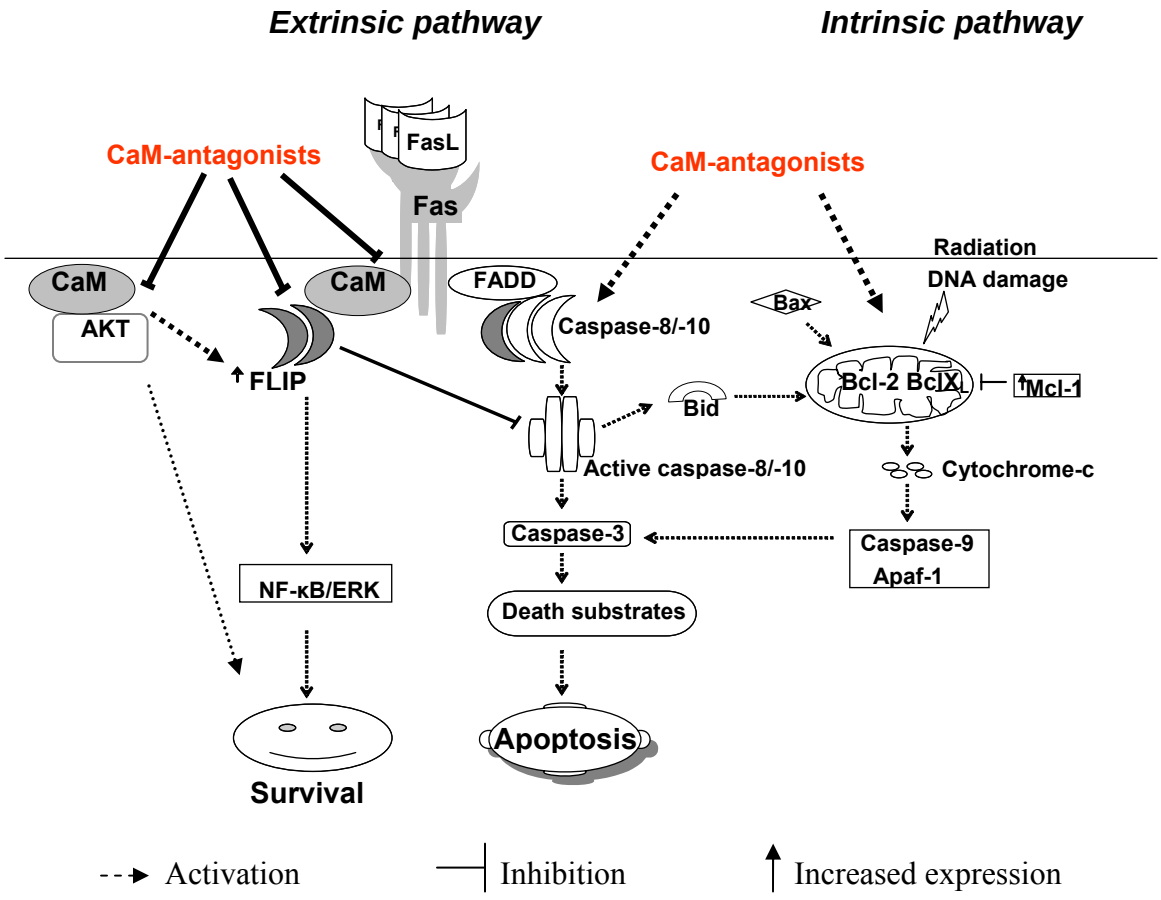


Figure 1. Regulators of apoptosis
Extrinsic pathway of apoptosis initiated by binding of Fas ligand (FasL) to its cognate receptor, Fas (highlighted in grey), is shown in the figure. Fas activation causes recruitment of FADD, caspase-8, c-FLIP and CaM (highlighted in grey) into the DISC. There is a cross talk between extrinsic and intrinsic pathways of apoptosis through caspase-8 mediated cleavage of Bid.

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* SEER Cancer Statistics Review 1975-2005

APPENDIX

University of Alabama at Birmingham
Institutional animal care and use committee
Notice of approval

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 20, 2007

TO: Jay M. McDonald, M.D.
LHRB 509 0007
FAX: 975-9927

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Cholangiocarcinoma Pathogenesis: Fas-Mediated Apoptosis
Sponsor: VA
Animal Project Number: 070507504

On May 30, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	24
Mice	B	48

Animal use is scheduled for review one year from May 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070507504 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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